Marzulli and Maibach's

Dermatotoxicology

Seventh Edition

Edited by

Hongbo Zhai

Klaus-Peter Wilhelm

Howard I. Maibach

CRC Press
Taylor & Francis Group

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Dedication

To

Francis N. Marzulli, Torkil Menne, and Ian White for their dedication to expanding dermatotoxicology's scientific and public health horizons.

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Preface

Dermatotoxicology—First edition, published in 1977, covered much of the embryonic field in 567 pages.

The seventh edition (2007)—consisting of 108 chapters—summarizes much of the current field, defining what was largely an intuitive science to today's often refined metrics and mechanisms.

Most chapters from previous editions have been revised. New chapters reflect the expanding science. Major areas include pharmacogenetics, racial differences, percutaneous penetration enhancers, sensitive skin, mechanisms of allergic contact dermatitis (ACD) and irritant contact dermatitis (ICD), occupational ACD, mechanisms in cutaneous drug hypersensitivity reactions, OECD guidelines, stratum corneum quantification, Kawai method for testing irritation, tests for sensitive skin, ACD threshold doses, ECVAM, corticosteroid-induced glaucoma, genotoxic activity, exogenous ochronosis, air bag injuries, cigarette and skin, chemical analysis of tattoo, anti-irritant, medical device regulation, diagnosis of drug hypersensitivity, water decontamination, hair in toxicology, textiles and human skin, *in vivo* human transfer of topical bioactive drug, fragrance allergens, ten genotypes of ICD, reconstituted human epidermis, functional map, and skin buffering.

Society (government, the media, the public, and the law) has become increasingly aware of the skin as an important route of systemic exposure to chemicals—adding impetus to the field of dermatotoxicology.

Unfortunately, in spite of the seventh edition's mass, much science was left out—so that editions one through six remain not relics—but sources of relevant information.

Our special thanks go to Patricia Roberson for the meticulous management of this complex volume. We welcome your critiques and suggestions for the eighth edition.

Hongbo Zhai
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1 Pharmacogenetics and Dermatology

Ernest Lee and Howard I. Maibach

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1.1 INTRODUCTION

Pharmacogenetics is the study of genetically determined variations in response to drugs in humans or in laboratory organisms.[1] For the clinician, this concept is relevant when asking why a drug is efficacious for one segment of the population, ineffective for another, and fatal or toxic to a third.[2] The spectrum of effectiveness depends on issues such as compliance, drug availability, proper dosing, and pharmacogenetics.

Considering the role of pharmacogenetics in dermatology, its influence is more commonplace than is probably realized. For example, we see its role even in a small degree in one of the most common reasons for an office visit to a dermatologist: acne. Numerous acne drugs exist, but efficacy rates are variable. The reasons for this are incompletely known: this disease tends to be multifactorial and it is difficult to isolate the purely genetic component.

Pharmacogenetics not only has a role in the efficacy of a drug in the population but also when considering the use of two drugs for a single disease entity. For chronic idiopathic urticaria, both loratadine and cetirizine are considered first-line therapy. Although some may benefit equally from both drugs, one drug or the other may be better for certain individuals due to differing genetic profiles.[3]

Another important area of pharmacogenetics is the concept of drug–drug interactions. This issue has gained increasing notoriety with the well-documented adverse cardiac effects of the antihistamines astemizole and terfenadine. In fact, many commonly used dermatologic drugs, can cause QT prolongation and *torsades de pointes*,[4] include antibiotics such as flouroquinolones, macrolides, and the imidazole antifungal agents. Roos and Merk have summarized important drug interactions in dermatology.[5]

The patients at greatest risk of adverse drug interactions (ADI) were stated by Andersen and Feingold as the following: those with impaired hepatic and renal function, the elderly, those with AIDS (acquired immunodeficiency syndrome), those who are acutely ill, those using prescriptions from several physicians, and those suffering from polypharmacy.[6]

What follows next are the classes of commonly encountered medications used in dermatology where pharmacogenetics plays an important role in its efficacy and safety.

1.2 AZATHIOPRINE

Advances have been made in understanding pharmacogenetics in the use of azathioprine in dermatology.[7] Eleven percent of the population has low thiopurine methyltransferase (TPMT) activity and is vulnerable to myelosuppression with azathioprine treatment.[8] Furthermore, one in 300 individuals has undetectable TPMT activity, and is susceptible to rapid-onset, prolonged, life-threatening pancytopenia if treated with conventional doses of azathioprine.[9] For over 40 years, azathioprine has been extensively used as an immunosuppressant. Most frequently it is used for the immunobullous disorders but occasionally for atopic dermatitis[10] also as a corticosteroid-sparing adjunctive therapy.[11] Much of the drug's pharmacokinetic and phamacodynamic characteristics are incompletely characterized despite widespread clinical experience. Most are aware of inactivation of 6-mercaptopurine (6-MP), the major azathioprine metabolite, by xanthine oxidase and the potential interaction with allopurinol, a xanthine oxidase inhibitor. However, fewer note the other routes of 6-MP metabolism and their relevance to toxicity and therapeutic response to azathioprine: namely TPMT and hypoxanthine-guanine phosphoribosyl transferase, which

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produce several active toxic thiopurine metabolites including 6-thioguanine nucleotides (6-TGN).

Objective evidence to support azathioprine efficacy is largely uncontrolled in nature: a search revealed two large, randomized, controlled studies that have attempted to evaluate efficacy in atopic dermatitis,[12,13] one for chronic actinic dermatitis,[14] one for bullous pemphigoid[15] and none for pemphigus vulgaris. The data on atopic dermatitis did support a role for azathioprine in the treatment of otherwise treatment-resistant disease. Of note is the study by Meggitt et al.,[12] who performed their study with dosing based on TPMT activity: patients with normal TPMT activity received 2.5 mg/kg/day of azathioprine whereas those with decreased TPMT activity received 1 mg/kg/day. There was a similar response to therapy in both groups of patients receiving azathioprine and no myelotoxicity was reported in the five azathioprine recipients with heterozygous TMPT activity. In the trial for chronic actinic dermatitis, marked improvement in the clinical status of actively treated patients led to early termination of the trial and the conclusion that oral azathioprine therapy is an effective and usually well-tolerated treatment in chronic actinic dermatitis. There was no consideration of differing enzyme levels between patients in this trial.

The data in the bullous pemphigoid trial did not support the dogma of a corticosteroid-sparing effect with azathioprine in bullous pemphigoid, but failure to consider the impact of interpatient variability on azathioprine metabolism and the adoption of a fixed-dosage regimen led to underdosage of some patients.[16] There is a need for adequately powered, prospective studies to determine efficacy and safety of azathioprine in pemphigoid and pemphigus. This should include measurement of the patient's TPMT activity and the regular monitoring of 6-TGN levels to confirm compliance and detect underdosage and toxicity. Enzyme levels need to be determined before dosing to avoid underdosing or toxicity, as well as monitoring adherence.

1.3 DAPSONE AND CYCLOSPORINE

Singer et al. review the interactions of cytochrome P-450 3A with dermatologic therapies.[17] Dapsone is one mainstay of dermatology whose metabolism is by the P450 3A class of hepatic microsomal enzymes. This drug has many uses, but in dermatology, it is used mostly in Hansen's disease (leprosy) and dermatitis herpetiformis. When concurrently used with rifampin, a P4503A inducer, it may result in as much as a 7–10-fold decline in dapsone levels. Dosage adjustments may be required in the treatment of such diseases as *Pneumocystis carinii* pneumonia, but not during concurrent rifampin therapy for leprosy because dapsone concentrations are still higher than the minimum inhibitory concentration.[18] Patients with glucose-6-phosphate dehydrogenase (G6PD) deficiency may have profound hemolysis during sulfone, dapsone, or sulfapyridine therapy, and those at risk of having the deficiency (blacks, Asians, and those of Mediterranean descent) should have a G6PD level ordered before starting therapy.[19]

Cyclosporin A is also a substrate for P450 3A. Therefore any drug that inhibits or induces this enzyme system can affect drug levels. Inducers P450 3A include the anticonvulsants carbamazepine, phenobarbital, phenytoin, the antituberculous agents rifampin and isoniazid, and drugs such as clotrimazole, griseofulvin, phenylbutazone, and dexamethasone.[20] Confirmed inhibitors include ketoconazole,[21] diltiazem, verapamil, progestrone, and the erythromycins.[22]

1.4 ANTIHISTAMINES

An important drug class with adverse interactions is the antihistamines, though these interactions are more significant from a historical perspective as terfenadine and astemizole have now largely been withdrawn from the market for pretreatment of chronic urticaria. Like loratadine, terfendaine and astemizole are metabolized by the major human liver enzyme, CYP3A4. Any drug that induces or inhibits this enzyme can affect drug levels. When these aforementioned medications were still on the market, the concurrent administration of terfenadine or astemizole with erythromycin, itraconazole, or ketoconazole had the potential to cause life-threatening ventricular arrhythmias such as *torsade de pointes*.[23] Cimetidine also has the potential to interact with the various drugs that use the P-450 metabolic pathway. By inhibiting this pathway, it can cause increased and potentially dangerous levels of warfarin, imiprimine, phenytoin, and theophylline.[24,25]

1.5 ANTIFUNGALS

Bickers summarized the potential interactions of antifungals with other classes of drugs.[26] Itraconazole is used for many molds such as *Aspergillus*, yeasts, and dermatophytes. Essentially, as a P450 3A3/4 inhibitor, any drug that uses this metabolic pathway is affected. Along with fluconazole and ketoconazole, it can increase levels of astemizole, cisapride, cyclosporin, dapsone, erythromycin, tacrolimus, and terfenadine.

Amphotericin B binds sterols in the fungal membrane. It is produced by *Streptomyces nodosus* as an amphoteric polyene macrolide. Its most common adverse effect is nephrotoxicity from an increased excretion of potassium leading to hypokalemia and renal tubular acidosis.[27] Because of this potential, this drug can further exacerbate hypokalemia caused by corticosteroids and digitalis glycosides.[28] Amphotericin B may also act synergistically with flucytosine by augmenting fungal membrane penetration.[29] The newer oral antifungal agent terbinafine has important drug interactions with caffeine, cimetidine, cyclosporine, nortriptyline, rifampin, theophylline, and warfarin.[30]

1.6 ANTIBIOTICS

Some macrolides and fluoroquinolones inhibit hepatic enzymes. P450 enzymes are inactivated by erythromycin[31] and can cause high levels of benzodiazepines, warfarin, cyclosporin A, and

theophylline on coadministration. Clarithromycin has fewer interactions and azithromycin does not seem to cause adverse drug interactions with the above.[32] With fluoroquinolones, important drug interactions include antacids, iron, sucralfate, cimetidine, caffeine, cyclosporine, hydantoins, anticoagulants, and theophylline.[33] For example, seizures and other important interactions have been reported with theophylline.[34] However, each drug within this class has its own safety profile. While enoxacin and ciprofloxacin inhibit some hepatic cytochrome P-450s, ofloxacin has little or no effect on theophylline clearance.[35]

Of the tetracyclines, P-450 enzymes metabolize only doxycycline.[36] Therefore, enzyme inhibitors or inducers will affect it. Tetracyclines may cause digitalis toxicity by interacting with digoxin possibly through effect on the bowel flora.[37] Coadministration of minocycline and amitriptyline may accelerate cutaneous pigmentation.[38]

Potential ADIs with antibiotics and oral contraceptives are of great relevance in dermatologic practice. The enterohepatic circulation of contraceptive steroids can be interfered with by antibiotic effects on bacterial flora in the bowel, and lower serum levels of the contraceptives can result. Some have suggested increasing the estrogen component of the pill to 50 μg or adding other forms of birth control for the duration of antibiotic therapy.[39] However in practice, the failure of oral contraceptives with oral antibiotics is low.[40] In fact, a recent review of the literature suggests that there is little convincing evidence to show a systematic interaction between antibiotics and oral contraceptives other than rifampin.[41]

1.7 CONCLUSION

Pharmacogenetics is and will continue to be an important concept in clinical medicine. Its importance in dermatology is reflected in the increasing number of articles being written on this subject. We refer to reviews by Lowitt and Shear[42] or Ameen et al.[43] for further reading. In addition, websites exist that summarize important P450 drug interactions.[44] We provide a detailed evidence-based description of this literature—in a manner that permits the health care provider the tools to understand what is and is not known. Dermatology, with its numerous drug therapies, will certainly intertwine with pharmacogenetics as it researches and develops its pharmacologic arsenal.

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2 Ethnic Differences in Skin Properties: Objective Data

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2.1 INTRODUCTION

Ethnic differences in skin properties may explain disparities seen in dermatologic disorders and provide insight into appropriate differences in the management of these disorders. However, ethnic differences in skin have been minimally investigated by objective methods and the data are often contradictory. The current experimental human model for skin is largely based upon physical and biochemical properties known about Caucasian skin. Thus, anatomical or physiological properties in skin of different races that may alter a disease process or treatment of that disease are not being accounted for.

Early studies show similarities in black and white skin. For example, Thomson[77] and Freeman et al.[27] conclude that the stratum corneum (SC) is of equal thickness in blacks and whites. However, in 1974, Weigand et al.[85] demonstrated a difference in black and white skin with regard to a variable other than color. They demonstrated that the SC of black skin contains more cell layers and black skin requires more cellophane tape strips to remove the SC than white skin. Greater variability in the number of tape strips used within the black subject pool was also found, compared with the white subject pool, but this variability was not correlated with degree of skin pigmentation. The mechanisms behind greater intercellular adhesion among black individuals may involve lipids,[10] because the lipid content of the SC ranges from 8.5 to 18.4%, with higher values in blacks.[61] Since SC thickness is believed to be equal,[87] the data reflected greater intercellular adhesion among the black individuals.[85] Recently developed quantitative techniques for determining SC mass are yet to be utilized for this purpose.[24]

While Weigand et al.[85] objectively demonstrated a different physical property in black and white skin, some other studies demonstrating differences used more subjective methods. For example, erythema has been used as a measure of demonstrating skin irritation.[6,46,85] Since erythema is difficult

Adapted from NO Wesley and HI Maibach, Racial (ethnic) differences in skin properties: the objective path, *American Journal of Clinical Dermatology*, 4(12), 843–860, 2003. With permission.

to assess in a person with dark skin, such subjective methods are not sufficient in evaluating ethnic disparities. Thus, to accurately report ethnic differences in skin properties, objective methods should be utilized. Objective measurements that have been studied are transepidermal water loss (TEWL), water content (WC) (via conductance, capacitance, resistance, and impedance), corneocyte variability, blood vessel reactivity, elastic recovery/extensibility, pH gradient, lipid content, surface microflora, electron microscopy and immunoelectron microscopy of mast cell granules, confocal microscopy of epidermal innervation, microtopography, sebaceous function, vellus hair follicle distribution, morphology and distribution of melanosomes, and resistance to photodamage.

Given the increasing ethnic diversity in the United States, it is essential to clarify relationships among race, color, ethnicity, and disease process. Even though these objective methods have been used to compare skin of different races, the data that exist remain minimal. Additionally, the data are often confusing and difficult to interpret. We explore and attempt to clarify the objective data available in differentiating skin properties of different races. Objective definitions of skin color are yet to be established. We introduce certain objective differences that have been established to date. We searched MEDLINE®, MD Consult, Science Citations Index, the Melvyl Catalogue in the CDL-Hosted Database of University of California, San Francisco, California, Yahoo, Google, standard dermatology textbooks, and University of California, San Francisco, California surge building library files from 1967 to August 2006. Keywords in searches included words pertaining to race (i.e., race, ethnicity, black, African, white, Caucasian, Asian, Hispanic) and dermatology (i.e., skin, skin physiology, skin function). The references of each study were then reviewed for other studies that examined ethnic differences with objective methods. Studies pertaining to ethnic differences in hair were excluded to keep the review focused on skin function/physiology. Words used to describe race/ethnicity of study individuals are the same as those used by the authors in the respective texts.

2.2 TRANSEPIDERMAL WATER LOSS

One role of the skin is to maintain an effective barrier against loss of body fluids and absorption of externally applied substances.[54] The total amount of water vapor passing the skin can be divided into water vapor passing the SC by passive diffusion and water vapor loss as a result of sweating.[66] Baseline water diffusion (imperceptible or unnoticed perspiration) amounts to 2.25 $\mu L/m^2$/sec and is distinct and separate from sweat gland secretion.[41] Originally, the term TEWL was used to indicate the amount of water vapor passing through the SC by passive diffusion.[66] Current literature, however, refers to TEWL as the total amount of water vapor loss through the skin and appendages, under nonsweating conditions.[66] Therefore, note that TEWL is a true reflection of SC barrier function only when there is no sweat gland activity. In addition to characterizing the water barrier function of skin, measurement of TEWL has been utilized widely in studies to perform predictive irritancy tests and to evaluate the efficacy of therapeutic treatments on diseased skin.[23] To date, TEWL is the most studied objective measure in defining differences between the skin of different ethnicities.

Wilson et al.[87] demonstrated higher *in vitro* TEWL values in black compared with white skin. Water evaporation measured from skin taken from 10 African-American and 12 Caucasian cadavers matched for age and gender was then converted into TEWL using an equation. In addition to finding differences in black and white skin physiology, the investigators also found that the TEWL of both races increased with skin temperature. These results were explained on the basis of a prior *in vivo* study from 1941 showing that blacks had a lower skin and rectal temperature during exercise.[65] Thus, in maintaining equal temperatures between black and white skin, they concluded that it would be expected that black skin would have a greater rise in temperature to achieve the same endpoint temperature and therefore a higher TEWL.[87] Although comparisons between *in vitro* and *in vivo* studies are frequently made in medicine, note that the *in vitro* study may not have accounted for some physiological functions, such as sweating. Also, accounting for physiologic temperature differences by race in skin may be difficult in an *in vitro* study. Since TEWL depends on passive water vapor loss, and based on laws of physics regarding passive diffusion, the rate of water vapor diffusion across the SC is theoretically directly related to the ambient relative humidity and temperature,[5] then, it is reasonable to assume that the increased TEWL in black skin is associated with an increase in temperature if, in fact, a difference in black and white skin temperature does exist.

A subsequent *in vivo* study by Berardesca and Maibach[7] supported the findings of the *in vitro* study. The investigators determined the difference in irritation between young black and white skin. They applied 0.5 and 2.0% sodium lauryl sulfate (SLS), a water-soluble irritant (surfactant), to untreated, preoccluded, and predelipidized skin and quantified the resulting level of irritation using WC, TEWL, and laser Doppler velocimetry (LDV) of the SC. No statistical difference was found in irritation between the two groups based on WC and LDV, however, a statistical difference in the TEWL results of 0.5% SLS applied to the preoccluded skin was found. In that test, blacks had 2.7 times higher TEWL levels than whites ($p < 0.04$), suggesting that blacks in the preoccluded state are more susceptible to irritation than whites. This theory opposes the traditional clinical view, based on observing erythema,[6] that blacks are less reactive to irritants than whites.

Berardesca and Maibach[8] used the same model to compare differences in irritation between Hispanic and white skin. Although there were no significant differences in TEWL, WC, or LDV between the groups at baseline, the data showed higher values of TEWL for Hispanics compared with whites after SLS-induced irritation. However, these values were not statistically significant. The investigators noted that the reaction of Hispanic skin to SLS resembles that of black skin when irritated with the same substance.[7] Since skin

pigmentation varies greatly within the Hispanic and black communities, the degree of skin pigmentation, according to Fitzpatrick's model,[25] could represent an important variable.

However, in a later study, Berardesca et al.[12] found no significant difference *in vivo* in TEWL between race or anatomic site for baseline observation. The investigators examined 15 blacks, 12 whites, and 12 Hispanics to account for degree of skin pigmentation, matched for age and gender, and measured TEWL, WC (via skin conductance), skin thickness, and biomechanical properties, such as skin extensibility, at two sites, the dorsal and volar forearm. Skin sites that vary in sun exposure were used to highlight the protective effects of melanin from ultraviolet (UV)-induced damage. Ethnic differences in skin conductance (blacks $>$ whites) and skin elasticity were found and are discussed in Sections 2.3 and 2.6. However, even though the investigators expected a higher TEWL in blacks, based on previous studies[7,87] and based on a higher WC (skin conductance) in blacks found in their current study, no significant difference in TEWL was found between races or anatomic sites. They accounted for the higher WC in black skin with no ethnic differences in TEWL on the basis that black skin might have increased intercellular cohesion[85] and increased lipid content[61] keeping the water in.

In contrast, Kompaore et al.[39] found significantly higher TEWL values in blacks and Asians compared with whites. After the application of methyl nicotinate (a vasodilator), the investigators evaluated TEWL and lag time to vasodilatation by LDV, before and after removal of the SC by tape stripping. The participants were seven black men, eight white subjects (six male and two female), and six Asian men all living in France, aged 23–32 years, without skin disease. Before tape stripping, TEWL was 1.3 times greater in blacks and Asians compared with whites ($p < 0.01$); no difference was found between blacks and Asians. After 8 and 12 tape strips, TEWL values were highest in Asians overall (Asians 1.7 times greater than whites) ($p < 0.05$). The investigators concluded that, similar to previous studies,[7,87] skin permeability measured by TEWL was higher in blacks than in Caucasians. However, they also concluded that Asian skin had the highest permeability among the groups studied. Although the methods of this study were impressive and well documented, this finding has not yet been duplicated.

Sugino et al.[70] (abstract only) also included Asians in their study but found that baseline TEWL was, in decreasing order, blacks $>$ Caucasians $>$ Hispanics $>$ Asians. Aramaki et al.[3] compared TEWL, SC hydration, sebum secretion, laser Doppler flowmetry, content of melanin, and erythema on forearm at baseline and after SLS-induced irritation in 22 Japanese women (mean age 25.84 years) and 22 German women (mean age 26.94 years). There were no significant differences in TEWL between Japanese and German women before or after SLS stress. Another study (unpublished data) referenced in a review article[88] about Asian skin, compared TEWL in Asians and Caucasians and also found no statistically significant differences at baseline or after tape stripping; however, no vasoactive substance was applied.

In an attempt to compare degree of skin pigmentation as opposed to race, Reed et al.[59] compared 7 subjects with skin types V and VI (4 African Americans, 2 Filipinos, and 1 Hispanic) to 14 subjects with skin types II and III (6 Asians and 8 Caucasians). The investigators used TEWL to assess the ability of the SC to withstand or recover from insults to the epidermal permeability barrier (i.e., tape stripping). Subjects with skin type V/VI required more tape strippings (66.7 ± 6.9) compared to skin type II/III (29.6 ± 2.4) to achieve the same TEWL, i.e., skin type V/VI had increased barrier strength (integrity). These findings correlate with those of Weigand et al.[85] that black skin has more cell layers and increased intercellular adhesion. Furthermore, it was also found that water barrier function (measured by TEWL) in skin type V/VI recovered more quickly. This study demonstrated differences in SC barrier function as measured by TEWL between different skin types possibly independent of race. Since the sample size with skin types V and VI was small, further studies with larger sample sizes should be conducted to support these findings.

Warrier et al.[83] recognized the discrepancies in data comparing skin of blacks and whites. Thus, in an attempt to clarify the data, the investigators studied TEWL, electrical capacitance, skin pH, elasticity, dryness/scaling, and skin microflora in 30 black and 30 white women, aged 18–45 years. In contrast to all previous studies which found an increase in TEWL in blacks compared with whites,[7,39,87] Warrier et al.[83] found TEWL to be significantly lower on the cheeks (20% less) and legs (17% less) in blacks compared with whites ($p < 0.05$). TEWL was also lower on the forearms in blacks, but this was not statistically significant. Prior studies examined the forearm, inner thigh, and back. Does the anatomic site act as a confounding variable in obtaining TEWL values? In a study on Caucasian subjects, TEWL values of the posterior auricular and forehead SC were higher than SC of the arm, forearm, or the abdomen.[67] Thus, perhaps there are also differences in TEWL when comparing the sites examined (cheeks and lower legs) by Warrier et al.[83] to those of prior studies (forearm, inner thigh, and back).[7,8,12,39,87] Although this study used a larger sample size, the discrepancy in data warrants further studies with large sample sizes and comparisons of various anatomic sites.

Berardesca et al.[11] examined differences in TEWL as well as pH in 10 Caucasian (skin types I and II) and 8 black African-American (skin type VI) women at baseline and after tape strippings. TEWL increased for both races with each tape stripping. Interestingly, even though black women had a higher TEWL at baseline and after each tape stripping compared with Caucasian women, the differences were only statistically significant (1.2 times greater) after three ($p < 0.05$) and six ($p < 0.03$) tape strips. Similar to the study by Reed et al.,[59] it was also found that recovery of water barrier function, as measured by TEWL 48 h after stripping, was greater in blacks as compared with Caucasians, but the difference was not statistically significant.

Tagami[74] provided additional information on Asian skin by comparing TEWL between 120 Japanese and 322 French

women, aged 20–70 years. The skin type of the French women was not specified. His research team measured TEWL on cheeks and midflexor surface of forearms of all subjects, under the same environmental conditions. Although, TEWL was lower in Japanese women, the data were not statistically significant. These findings further supported those of Aramaki et al.[3]

Hicks et al.[33] grouped patients on the basis of skin color (Fitzpatrick skin type), as in Reed et al.,[59] in studying the difference between susceptibility of black and white skin to irritant contact dermatitis (ICD). The 14 participants were grouped as 8 whites (skin types II/III) and 6 blacks (skin types V/VI), between the ages of 18 and 40 years. The skin on the volar forearm was exposed to 4 and 1% SLS and evaluated by reflectance confocal microscopy, TEWL, laser Doppler velocimetry, and routine histology at 6, 24, and 48 h after initial application. Changes in TEWL and SC thickness after exposure to 4% SLS at 48 h were negatively correlated in both groups. White participants showed a trend toward greater mean increases in TEWL after SLS exposure than black participants, supporting the possibility that the barrier function in black skin is more durable than white skin, but the differences were not statistically significant. Overall, results from all methods of evaluation suggested reduced susceptibility of black skin to ICD. However, while there was no significant difference between SC thickness of control sites in both groups (consistent with Weigand et al.), the SC thickness was significantly less in blacks as compared to whites after exposure to 4% SLS at 48 h ($p < 0.05$). This pattern of SC thinning seems to contradict the findings of reduced susceptibility of black skin to ICD. A larger sample size may be necessary to clarify this discrepancy and achieve a statistically significant trend in TEWL changes.

In another evaluation of differences between African American and white skin, Grimes et al.[29] did not find significant differences in TEWL *in vivo*. The subjects consisted of 18 African American and 19 white adults between the ages of 35 and 65, with a subset of 8 (3 black, 5 white) participating in chemical challenge of 5% SLS. Methods of evaluation included clinical evaluation and instrumental measurements of sebum level, pH, moisture content, and TEWL. Although there were differences in visual assessment of photoaging and hyperpigmentation, the baseline instrumental findings from all methods indicated no significant differences between African American and white skin. Within 6 h of irritation, there was a significant change in TEWL in white participants; however, after 24 h, TEWL in both groups was similar. Owing to the small sample size of the chemical challenge subset, statistical analysis on these data was not performed. The overall findings of this study support the postulation that, objectively, there is little difference between African American and white skin. However, again based on small sample size, it is difficult to make definitive conclusions based on the data.

Pershing et al.[53] found a significant difference in TEWL between Caucasians and Asians with topical application of capsaicinoids. The study measured TEWL, skin surface temperature, and erythema after application of various capsaicinoid analogs at various concentrations on volar forearms of six Caucasians and six Asians, aged 19–63 years. The results were baseline adjusted, control site-corrected over time for each parameter to normalize data for inherent differences among skin sites. Increasing concentrations of total capsaicinoid were not associated with a proportional change in TEWL in either Caucasians or Asians. However, a capsaicinoid concentration of 16 mg/mL produced statistically less TEWL in Asians than Caucasians ($p < 0.05$); specifically, there was an increase of the mean TEWL in Caucasians but a decrease in Asians. The investigators concluded that changes in TEWL between Caucasians and Asians with capsaicinoids, but not irritants (such as SLS in Aramaki et al.[18]), may reflect the effect of vehicle composition (isopropyl alcohol for capsaicin versus water for irritants) or other physiologic skin functions (such as cutaneous blood flow) in determining TEWL.

Astner et al.[4] evaluated ethnic variability in skin response to a household irritant (Ivory dishwashing liquid) by applying the irritant to the anterior forearms of 15 Caucasian subjects and 15 African-American subjects. The investigators observed significantly higher mean values for TEWL in Caucasians compared to African Americans ($p \leq 0.005$) like Warrier et al. had found previously. The 30 participants were patch tested to graded concentrations of Ivory soap and evaluated with clinical scoring, reflectance confocal microscopy, TEWL, and fluorescence excitation spectroscopy. There was a positive, dose-dependent correlation between TEWL values and irritant concentration in all groups. However, not only was the mean TEWL higher in Caucasians, but the relative increment of increase in response to the graded irritant concentrations were also higher in Caucasians when compared to African Americans ($p \leq 0.005$). The researchers suggested the lower values of TEWL in African Americans in this study may reflect the greater intercellular cohesiveness in African-American skin (Weigand et al.[84]).

While the data regarding TEWL (summarized in Table 2.1) are conflicting, the overall evidence, except for the 1991 study by Berardesca et al.,[12] and later, the studies by Hicks et al.[33] and Grimes et al.,[29] supports some difference between black and Caucasian skin. Most studies using the forearm, back, and inner thigh[7,11,39,59,70,87] showed a greater TEWL in blacks compared with whites; however, the only study that used a larger sample size, by Warrier et al.,[83] found TEWL to be less in blacks than whites when measured on the cheeks and legs. In addition, like Warrier et al., a smaller study by Astner et al.,[4] found the mean TEWL on forearms of whites to be greater than those on blacks after irritant stress. Perhaps, the anatomic site examined causes discrepancies in TEWL values. Also, TEWL measurements with regard to Asian skin may be deemed inconclusive as baseline measurements have found Asian skin to have TEWL values that are equal to black skin and greater than Caucasian skin,[39] less than all other ethnic groups,[70] and no different than other ethnic groups.[3,74,88] Additionally, Pershing et al.[53] found an increase in TEWL of Caucasians but a decrease in TEWL of

TABLE 2.1
Transepidermal Water Loss (TEWL)[a]

Study	Technique	Subjects	Site	Results
Wilson et al.[87]	*In vitro*	Blacks 10 (mean age 38.6 y) Caucasians 12 (mean age 41.1 y)	Inner thigh	• TEWL blacks 1.1× > Caucasians (mean corrected log TEWL 2.79 and 2.61 μg/cm²/h, respectively) ($p < 0.01$ for both values)
Berardesca et al.[7]	*In vivo*—topical application of SLS (irritant)	Black men 10 (age 29.9 ± 7.2 y) White men 9 (age 30.6 ± 8.8 y)	Back	• No significant difference in TEWL between blacks and whites at baseline **After SLS stress** • TEWL blacks (untreated, preoccluded, and predelipidized) > whites but only statistically significant (2.7× greater) for 0.5% SLS applied in the preoccluded area ($p < 0.04$)
Berardesca et al.[8]	*In vivo*—topical application of SLS (irritant)	Hispanic men 7 (age 27.8 ± 4.5 y) White men 9 (age 30.6 ± 8.8 y)	Upper back	• No significant differences in TEWL between Hispanics and whites at baseline **After SLS stress** • TEWL Hispanics (untreated, preoccluded, and predelipidized) > whites, but not statistically significant
Berardesca et al.[12]	*In vivo*	Blacks 15 (mean age 46.7 ± 2.4 y) Whites 12 (mean age 49.8 ± 2 y) Hispanics 12 (mean age 48.8 ± 2 y)	Volar and dorsal forearm	• No significant difference in TEWL between site or ethnicity at baseline
Kompaore et al.[39]	*In vivo*—topical application of MN-vasodilator	Blacks 7 Caucasians 8 Asians 6 (age 23–32 y, all)	Volar forearm	**MN given before tape stripping** • TEWL blacks and Asians 1.3× > Caucasians ($p < 0.01$); no difference between blacks and Asians **After 8 and 12 tape strips** • TEWL Asians > blacks > Caucasians ($p < 0.05$) (Asians 1.7× greater than Caucasians)
Sugino et al.[70]	*In vivo*	Blacks, Caucasians, Hispanics, Asians (no. of subjects, ages not specified)	Not documented	• Baseline TEWL blacks > Caucasians ≥ Hispanics ≥ Asians
Reed et al.[59]	*In vivo*	Skin type V/VI: African American 4 Filipino 2 Hispanic 1 Skin type II/III: Asian 6 Caucasian 8 (age 22–38 y, all)	Volar forearm	• Skin type V/VI required more tape strippings (66.7 ± 6.9) compared to skin type II/III (29.6 ± 2.4) to achieve the same TEWL, i.e., skin type V/VI had increased water barrier strength (integrity) • Barrier function in skin type V/VI recovered more quickly
Warrier et al.[83]	*In vivo*	Black women 30 White women 30 (age 18–45 y, all)	Left and right medial cheeks, midvolar forearms, lateral midlower legs	• TEWL blacks < whites on cheeks (20% less) and legs (17% less) at baseline ($p < 0.05$); also lower on forearm but not statistically significant
Berardesca et al.[11]	*In vivo*	Black women 8 Caucasian women 10 (mean age 42.3 ± 5 y, both)	Midvolar forearm	**After tape stripping** • TEWL blacks 1.2× > Caucasians after 3 ($p < 0.05$) six tape strips ($p < 0.03$)
Aramaki et al.[3]	*In vivo*—topical application of SLS (irritant)	Japanese women 22 (mean age 25.84) German women 22 (mean age 26.94)	Forearm	• No significant difference at baseline or after SLS stress
Tagami[74]	*In vivo*	Japanese women 120 French women 322 (age 20–70 y, all)	Cheeks and midflexor forearm	• TEWL Japanese < whites but not statistically significant

(*continued*)

TABLE 2.1 (continued)
Transepidermal Water Loss (TEWL)[a]

Study	Technique	Subjects	Site	Results
Hicks et al.[33]	*In vivo*—topical application of 1 and 4% SLS (irritant)	White: Skin Type II 6 Skin Type III 2 Black: Skin Type V 5 Skin Type VI 1 (age 18–40 y, all)	Volar forearm	• TEWL whites > blacks but not statistically significant
Grimes et al.[29]	*In vivo*—topical application of 5% SLS (irritant)	African American 18 White 19 (age 35–65 y, women, all) SLS stress: African American 3 White 5	Inner forearm	• Baseline: No significant difference • After SLS stress: Immediate increase in TEWL of white subjects, but increase no longer evident after 24 h and found to be similar to African Americans (not statistically significant)
Pershing et al.[53]	*In vivo*—topical application of capsaicinoid analogs	Caucasians: Male 3 Female 3 Asians: Male 3 Female 3 (age 19–63 y, all)	Volar forearm	• Increasing concentrations of total capsaicinoid not associated with proportional change in TEWL, in all subjects. • Capsaicinoid concentration of 16 mg/mL produced ↑ mean TEWL in Caucasians, ↓ mean TEWL in Asians ($p < 0.05$)
Astner et al.[4]	*In vivo*—topical application of Ivory soap (irritant)	Caucasians 15 (Skin type II/III) African Americans 15 (Skin type V/VI) (age 18–49 y, all)	Anterior forearm	• Positive dose-dependent correlation between TEWL and irritant concentration: Mean TEWL Caucasians > African Americans ($p \leq 0.005$) • Relative increment of increase in TEWL after irritant: Caucasians > African Americans ($p \leq 0.005$)

Note: MN = methyl nicotinate; SLS = sodium lauryl sulfate; y = years.

[a] All of the evidence supports TEWL blacks > whites, except for Berardesca et al.,[12] Hicks et al.,[33] and Grimes et al.[29] who all found no significant difference; and Warrier et al.[83] and Astner et al.[4] who found blacks < whites. TEWL measurements of Asian skin are inconclusive as they have been found to be equal to black skin and greater than Caucasian skin (Kompaore et al.[39]), equal to Caucasian skin (Aramaki et al.[3] and Tagami[74]), and less than all other ethnic groups (Sugino et al.[70]). Pershing et al.[53] found an increase in TEWL of Caucasians but a decrease in TEWL of Asians in response to high concentrations of topical capsaicinoids.

Source: Wesley, N.O. and Maibach, H.I., *Am. J. Clin. Dermatol.*, 4(12), 843–860, 2003. With permission.

Asians in response to high potency capsaicinoids, the results of which are difficult to categorize.

If water barrier function truly depends on degree of pigmentation, this has implications as to whether the SC gains or loses barrier integrity in cases of acquired hyper- or hypopigmentation. Further, differences in barrier integrity/function, as measured by TEWL, also has implications in the ability of people with different skin types and colors to withstand and recover from environmental insults as well as the ability to absorb topical therapeutic agents. Furthermore, TEWL may vary under different pathologic and physiologic conditions. Thus, the health and physiologic state of the subjects should be noted in future studies.

2.3 WATER CONTENT

WC or hydration of the skin can be measured by several methods including skin capacitance, conductance, impedance, and resistance. The use of capacitance to measure WC is based on the high dielectric constant of water compared with other substances.[22] Conductance is also based on the changes in the electrical properties of the SC when the skin is hydrated.[79] Dry SC is a medium of weak electrical conduction, while hydrated SC is more sensitive to the electrical field.[22] Resistance is the reciprocal of conductance. In general, skin capacitance and conductance show similar behavior with regards to measuring WC of the skin, while resistance and impedance are opposite. Possible sources of error or variation in measurement include sweat production, filling of the sweat gland ducts, the number of hair follicles, the electrolyte content of the SC, and artifacts from applied topical agents.[79]

In 1962, Johnson and Corah[34] found that blacks had higher levels of skin resistance at baseline than whites ($p < 0.01$) at two different laboratories in St. Louis, Missouri and San Diego, California. The St. Louis study examined 174 children

(22 black boys, 32 black girls, 65 white boys and 55 white girls), aged 83–92 months, while the San Diego study examined 42 subjects (16 black men, 5 black women, 16 white men, and 5 white women), mean age 23 years. While the investigators in this study did not correlate their measurements of skin resistance to WC in the skin, knowing the relationship of skin resistance to WC, we can deduce that a higher resistance in blacks may be correlated with a lower WC.

In addition to comparing TEWL, Berardesca and Maibach also compared WC by capacitance before and after topical administration of SLS in blacks and whites, and in another study, in Hispanics and whites.[7,8] There were no significant differences in WC between blacks and whites at baseline or after SLS stress.[7] In comparing Hispanics and whites, there was an increase in WC in Hispanics at baseline, but the difference was not significant; however, after SLS application, they found a significant increase in WC in Hispanics compared with whites when a negative visual score (i.e., no erythema) was given for irritation ($p < 0.01$).[8] In reviewing the data, however, we found that although the mean values for WC in Hispanics were greater than in whites, the standard deviations were also large. When an irritant reaction was visually detectable, the WC was proportionally increased in both races, eradicating a difference between them.

Berardesca et al.[12] examined WC by conductance on the volar and dorsal forearm of 15 blacks, 12 whites, and 12 Hispanics in addition to examining TEWL, skin thickness, and extensibility. Within each race studied, significant differences existed in WC between the volar and dorsal forearms (Table 2.2). Whites and Hispanics demonstrated decreased WC on the dorsal aspect of each arm compared with the volar side (22% less and 11% less, respectively), whereas blacks demonstrated a 13% decrease in WC on the volar aspect compared with the dorsal side. The differences, however, were statistically more relevant for white skin ($p < 0.001$) and less for blacks ($p < 0.02$) and Hispanics ($p < 0.05$). In comparing the races with each other, blacks and Hispanics had increased WC compared with whites on the dorsal forearm. On the volar forearm, however, Hispanics demonstrated greater WC than blacks and whites. Their findings do not correlate with those of the prior studies; however, this study measured WC at baseline using conductance, whereas the prior studies measured WC at baseline using resistance,[34] and at baseline and after SLS stress using capacitance.[7,8] The variability in WC observed between site and race are difficult to interpret. The investigators noted, however, that the white subjects had an increased amount of hair on the forearms compared with the other two groups, possibly accounting for some differences in the results.

Sugino et al.[70] measured WC with an impedance meter in blacks, whites, Hispanics, and Asians. They found that WC was highest in Asians compared with Caucasians, blacks, and Hispanics. The exact values and study size were not documented. The investigators correlated high WC with high ceramide and low TEWL values also measured in their study.

Warrier et al.[83] examined WC by capacitance at baseline in 30 black and 30 white women, aged 18–45 years. Black women had a significantly higher WC on the cheeks ($p < 0.05$), but there were no significant differences at baseline between blacks and whites on the forearms and the legs. They proposed that the difference found on the cheeks might be related to evidence of more elaborate superficial vasculature and more apocrine and mixed eccrine–apocrine glands in facial skin of blacks,[48] as well as on differences in melanin content, the packaging of melanocytes, and their ability to prevent epidermal photodamage.[26,35,49]

Manuskiatti et al.[45] studied seven black and five white women (mean age 25.8 ± 4.2 years) and five black women and five white women (mean age 64.7 ± 3.8 years) and measured WC (by capacitance) as well as desquamation index, as a measure of skin scaling, on the preauricle, posterior neck, dorsal upper arm, dorsal forearm, volar forearm, lower back, abdomen, thigh, and lower leg. The results of desquamation index are discussed in Section 2.4. They found no ethnic differences in WC, but did find significant differences between the younger and older women (younger had higher WC than older women).

Sivamani et al.[68] compared differences in friction coefficient, impedance, and amplitude/mean calculation of friction coefficient curves between Caucasian, African-American, Hispanic, and Asian subjects. Participants included 22 Caucasians, 14 African-Americans, 14 Hispanics, and 9 Asian volunteers aged 18–60 years. In addition to measuring baseline differences, the researchers assessed differences in response to polyvinylidene chloride occlusion, topical petrolatum, and topical glycerin applied to the volar forearm, based on gender, age, and ethnicity. Baseline measurements showed no significant differences in impedance between age, gender, or ethnicity. Notably, although there were no significant differences between right and left forearms, significant baseline variation was found between the distal and proximal volar forearms; the proximal forearms showed lower impedance than the distal forearms ($p < 0.001$). As impedance is a measure of WC, we can infer baseline differences in WC among anatomic sites from this study. Additionally, all interventions produced decreases in impedance from baseline (degree of decrease varied by intervention), but no significant differences between age, gender, or ethnicity. The authors concluded that there is little variation in volar forearm skin across gender, age, and ethnicity, providing an adequate site for testing of skin and cosmetic products.

Grimes et al.[29] measured baseline moisture content on the inner forearms of 18 African American and 19 white women, aged 35–65 years, based on capacitance. The study found no significant variation in baseline moisture content between African-American and white subject inner forearms.

The WC results of each study are summarized in Table 2.2. While Johnson and Corah[34] did not correlate resistance to WC in their study, it can be implied from their data that ethnic variance was found in WC. However, the SLS-induced irritation studies by Berardesca and Maibach[7,8] revealed no significant differences in WC between the races at baseline or after SLS stress, except for a questionable difference (high standard deviations) of Hispanics having greater WC than whites after SLS stress. Since it is believed that artifacts from

TABLE 2.2
Water Content[a]

Study	Technique	Subjects	Site	Results
Johnson and Corah[34]	*In vivo*—resistance	**St. Louis study**: Black boys 22; black girls 32 White boys 65, white girls 55 (age 83–92 months, all) **San Diego study**: Black men 16, black women 5 White men 16, white women 5 (mean age 23 y, all)	First and third fingers of right hand	• Skin resistance: Blacks > whites at baseline ($p < 0.01$), i.e., blacks have lower water content
Berardesca et al.[7]	*In vivo*—topical application of SLS (irritant)—capacitance	Black men 10 (age 29.9 ± 7.2 y) White men 9 (age 30.6 ± 8.8 y)	Back	• No significant differences between blacks and whites at baseline or after SLS stress
Berardesca et al.[8]	*In vivo*— topical application of SLS (irritant)—capacitance	Hispanic men 7 (age 27.8 ± 4.5 y) White men 9 (age 30.6 ± 8.8 y)	Upper back	• No significant differences between Hispanics and whites at baseline **After SLS stress** • Hispanics > whites when negative visual score was given for irritation ($p < 0.01$) (large standard deviations)
Berardesca et al.[12]	*In vivo*—conductance	Blacks 15 (mean age 46.7 ± 2.4 y) Whites 12 (mean age 49.8 ± 2 y) Hispanics 12 (mean age 48.8 ± 2 y)	Volar and dorsal forearm	• Blacks (13% less) volar < dorsal forearm ($p < 0.02$) • Whites (22% less) dorsal < volar forearm ($p < 0.001$) • Hispanics (11% less) dorsal < volar forearm ($p < 0.05$) • Blacks and Hispanics > whites on dorsal forearm at baseline • Hispanics > blacks and whites on volar forearm at baseline
Sugino et al.[70]	*In vivo*—impedance	Blacks, Caucasians, Hispanics, Asians (no. of subjects, ages not specified)	Not documented	• Asians > Caucasians, blacks, and Hispanics
Warrier et al.[83]	*In vivo*—capacitance	Black women 30 White women 30 (age 18–45 y, both)	Left and right medial cheeks, midvolar forearms, lateral midlower legs	• Blacks > whites on cheeks at baseline ($p < 0.05$) • No significant difference between two groups on forearms and legs
Manuskiatti et al.[45]	*In vivo*—capacitance	Black women 7 White women 5 (mean age 25.8 ± 4.2 y, both) Black women 5 White women 5 (mean age 64.7 ± 3.8 y, both)	Preauricle, postneck, dorsal upper arm, dorsal forearm, volar forearm, lower back, abdomen, thigh, lower leg	• No significant differences between blacks and whites at baseline
Sivamani et al.[68]	*In vivo*—impedance, topical application of petrolatum and glycerin	White 22 African American 14 Hispanic 14 Asian 9 (age 18–60 y, all)	Volar forearm	• Baseline: No significant differences in electrical impedance between age, gender, or ethnicity; impedance of proximal < distal forearm ($p < 0.001$) • After topical interventions: All interventions produced decrease in impedance; degree of decrease varied by intervention. No significant differences between age, gender, or ethnicity
Grimes et al.[29]	*In vivo*—capacitance	African American 18 White 19 (age 35–65 y, women, all)	Inner forearm	• Baseline: African Americans < whites, but not statistically significant

Note: SLS = sodium lauryl sulfate; y = years.

[a] Ethnic differences in water content, as measured by resistance, capacitance, conductance, and impedance are inconclusive.

Source: Wesley, N.O. and Maibach, H.I., *Am. J. Clin. Dermatol.*, 4(12), 843–860, 2003. With permission.

topically applied substances may alter values measured by capacitance or conductance,[22] this may play a factor in some of the values obtained by the SLS-irritant induced study. Additionally, Manuskiatti et al.[45] found no difference in WC between blacks and whites, but did find differences based on age. Berardesca et al.[12] and Warrier et al.,[83] however, did demonstrate ethnic variability in WC but the values varied by anatomic site. Sugino et al.[70] also demonstrated ethnic variability with Asians having a higher WC than other ethnic groups based on impedance. In contrast, Sivamani et al.[68] recently reported no ethnic differences in WC, both baseline and after various topical interventions, based on impedance; they did find variation of WC between different anatomic sites and with specific interventions. Of note, impedance, as used in the latter two studies, is less widely used than capacitance and conductance has been shown to be more sensitive to environmental and technical factors that affect the SC,[22] this makes it difficult to compare the results presented by these latter two studies. In another recent study using capacitance, Grimes et al.[29] showed no significant variation in baseline moisture content between African-American and white subjects inner forearms, further supporting studies by Berardesca and Maibach[7,8] and Manuskiatti et al.[45]

These findings, by measuring skin capacitance, conductance, impedance, and resistance, are difficult to interpret in terms of SC WC because other physical factors, such as skin microrelief, sweat production, and the presence of hair on the measuring site, may modify the quality of skin electrode contact.[22] Thus, it seems there may be factors other than race in the determination of WC and no conclusions with regard to race and WC can be made at this time. Studies with more subjects and the use of more than one method of measuring WC for accuracy should be considered in the future. In addition, since variation has been shown by anatomic sites, care should be taken to use consistent anatomical sites when comparing measurements of WC.

2.4 CORNEOCYTE VARIABILITY

Corneocytes differ in shape from the keratinocytes that produce them. The disk-like shape of corneocytes allows them to present with a large surface area in the horizontal position.[67] In Caucasians, the surface area of corneocytes differs by body site[55,67] and age.[42,67] It has also been demonstrated in Caucasians that corneocyte surface area is an important factor in the permeability of the skin to water loss and to percutaneous absorption of topically applied substances.[67]

Corcuff et al.[17] compared corneocyte surface area and spontaneous desquamation (via corneocyte count) on the upper outer arm in black African Americans, white Americans of European origin, and Asian-Americans of Chinese extraction. There were 18–25 age-matched subjects per group who were free from dermatological disorders. No difference in corneocyte surface area was found between the groups. However, spontaneous desquamation (corneocyte count) was increased in blacks by a factor of 2.5 compared with white and Asian skin ($p < 0.001$). The investigators felt that their findings were not consistent with earlier studies that showed increased intercellular adhesion[84] or increased TEWL[7,87] in black skin. This enhanced desquamation may (partially) account for "ashing" frequently seen clinically in black people.

In contrast, Warrier et al.[83] conducted a study that included corneocyte desquamation and had different results. The investigators studied 30 black and 30 white subjects, matched for age, and found that the desquamation index was greater on the cheeks and forehead of white subjects compared with black subjects. No difference was found on the legs. The investigators attributed the lower corneocyte desquamation on the cheeks and foreheads of blacks compared with whites to possible differences in moisturizing properties of sebum. These findings did not correlate with dry skin frequently seen clinically in black people. Since it is believed that corneocyte surface area varies by anatomic site in Caucasians,[67] perhaps corneocyte desquamation also varies by site. Corcuff et al.[17] studied the upper outer arm, whereas Warrier et al.[83] examined the cheeks, forearms, and lower legs. More studies of corneocytes desquamation should be conducted on the anatomic areas where dry skin is more frequently experienced. Additionally, the climate of the area where the study is done should be considered as it may influence desquamation. Warrier et al.[83] conducted their study over a 6-week period in winter, from December through February in Cincinnati, Ohio when temperatures and relative humidity are low and frequency of dry skin (winter xerosis) is high. In contrast, the city and climate are not documented in the study by Corcuff et al.[17]

In addition to measuring WC, Manuskiatti et al.[45] also examined the desquamation index in seven black and five white women (mean age 25.8 $\pm$ 4.2 years) and five black women and five white women (mean age 64.7 $\pm$ 3.8 years) on the preauricle, posterior neck, dorsal upper arm, dorsal forearm, volar forearm, lower back, abdomen, thigh, and lower leg. There were no differences in desquamation index between blacks and whites at all areas measured, except at the preauricular area ($p = 0.02$). However, whether blacks or whites had a higher desquamation index at this area was not specified. The investigators discounted the difference found at the preauricular area and attributed the difference to the small sample size used. Like the results found for WC, they also found significant differences in desquamation index based on age (older individuals had higher desquamation index than younger individuals at the preauricle). Overall, they concluded that age and anatomic site but not race demonstrate a significant influence on skin roughness and scaliness.

Overall, Corcuff et al.,[17] Warrier et al.,[83] and Manuskiatti et al.[45] reveal statistically significant results, but the findings are contradictory and therefore inconclusive (Table 2.3). Corcuff et al.[17] demonstrate greater corneocyte desquamation in blacks compared with whites on the upper outer arm. In contrast, Warrier et al.[83] found a greater desquamation index on the cheeks and forehead of whites compared with blacks. Additionally, Manuskiatti et al.[45] found a difference on the preauricular area only out of the numerous areas examined,

TABLE 2.3
Corneocyte Variability[a]

Study	Subjects	Site	Results
Corcuff et al.[17]	Black (mean age 33.5 ± 7.5 y) Caucasian (mean age 31 ± 7 y) Asian (mean age 26.5 ± 7.5 y) (18–25 subjects per group)	Upper outer arm	• No difference in corneocyte surface area • Spontaneous desquamation (corneocyte count) blacks 2.5× > Caucasians and Asians ($p < 0.001$)
Warrier et al.[83]	Black women 30 White women 30 (age 18–45 y, both)	Left and right medial cheeks, midvolar forearms, lateral midlower legs	• Desquamation index Blacks < Whites on cheeks (18% less) and forearms (20% less) ($p < 0.05$); but no significant differences on the legs
Manuskiatti et al.[45]	Black women 7 White women 5 (mean age 25.8 ± 4.2 y, both) Black women 5 White women 5 (mean age 64.7 ± 3.8 y, both)	Preauricle, postneck, dorsal upper arm, dorsal forearm, volar forearm, lower back, abdomen, thigh, lower leg	• No difference in desquamation index between blacks and whites except at preauricular area ($p = 0.02$) (which ethnicity greater not specified)

Note: y = years.

[a] Ethnic differences in corneocyte desquamation are inconclusive. The most clinically provocative observation is that of Corcuff et al.[17]—a 2.5 times greater spontaneous desquamation rate in blacks compared to Caucasians and Asians.

Source: Wesley, N.O. and Maibach, H.I., *Am. J. Clin. Dermatol.*, 4(12), 843–860, 2003. With permission.

but whether blacks or whites have a higher desquamation index is not specified.

Does the site of measurement of corneocyte desquamation, the WC and TEWL at that site, and the climate of the area where the study was done act as confounding variables for these results? In light of what is now known about WC and TEWL, the issue of corneocyte desquamation should be re-visited as these may be contributing variables. Corneocyte desquamation may have clinical implications in issues regarding the diagnosis and treatment of xerosis frequently seen in African Americans.

2.5 BLOOD VESSEL REACTIVITY

Cutaneous blood flow has been examined on numerous occasions to assess skin physiology, irritation, evaluation of dermatologic pathology/treatments, effects/delivery of drugs, and wound healing among other areas of interest.[82] The visual assessment of cutaneous microcirculation has been measured for centuries by the degree of erythema or pallor/blanching (visual scoring). However, the introduction of objective techniques for the evaluation blood flow has shown that the human eye is rather unreliable. Two techniques utilized by the papers to be discussed are LDV and photoplethysmography (PPG).

LDV is a noninvasive method that continuously follows the flow of red blood cells. It is based on measurement of the Doppler frequency shift in monochromatic laser light backscattered from moving red blood cells. It detects the frequency-shifted signal and derives an output proportional to the number of erythrocytes multiplied by their velocity in the cutaneous microcirculation.[50,82] LDV has been applied to skin physiology; diagnostics (especially scleroderma, Raynaud disease, and patch test reactions); predictive testing of irritancy (topical drugs, cosmetics, detergents, cleansing agents, products used in industry); and effects of drugs (vasodilators, minoxidil, sunscreens and UV light, topical corticosteroids [blanching]).[82]

PPG can be defined as the continuous recording of the light intensity scattered from a given source by the tissues and collected by a suitable photodetector.[13] Specific to the skin, it allows the registration of pulsative changes in the dermal vasculature and is synchronized with heartbeat. Infrared light from a transducer is absorbed by hemoglobin, and the backscattered radiation is detected and recorded. The backscattered light depends on the amount of hemoglobin in the skin, and the result obtained will therefore reflect the cutaneous blood flow. PPG has been used for studies of skin physiology, dermatological disorders, as well as systemic diseases.[82]

Guy et al.[31] enrolled six black subjects aged 20–30 years, six white subjects aged 20–30 years, and six white subjects aged 63–80 years, with good general health, no recent skin disease, and taking no prescription medications, and studied their response to topically applied vasodilator methyl nicotinate. The substance was applied to the volar forearm and blood vessel reactivity was measured by LDV and PPG. There was no significant difference between the ethnic groups in time to peak response, area under the response–time curve, or time for response to decay to 75% of its maximum value. However, the PPG maximum response was 40% less in the young black group than in the young white group ($p < 0.05$). The authors made note of the fact that the sensitivities of the two methods of study (LDV and PPG) were not

equivalent. They concluded that, overall, the data suggested a similarity in response among races and ages. The investigators did not discuss the significance or implications of the lower maximum PPG seen in the black subjects.

Berardesca and Maibach[7] performed a study to determine the difference in irritation between young black and Caucasian skin. They applied 0.5 and 2.0% SLS to untreated, preoccluded, and predelipidized skin and then quantified the resulting level of irritation using LDV, TEWL and WC of the SC. There were no significant differences between black and white skin for LDV at baseline or after application of SLS. The authors did note, however, that in blacks, application of the 0.5% SLS to untreated skin revealed minimal changes in cutaneous blood flow (as measured by LDV) compared with baseline. They used this finding to explain the decreased irritant-induced perceptible erythema in blacks.[85] However, after reexamining the data, we might consider that there was about the same degree of minimal change from baseline to application of 0.5% SLS in untreated skin in the Caucasian group.

Berardesca and Maibach[8] used the same model to compare differences in irritation between Hispanic and Caucasian skin. Like the SLS-induced irritation study comparing blacks and white, the same study comparing Hispanics and whites revealed equivalent blood vessel responses between the two groups.

Berardesca and Maibach performed a subsequent study using LDV, but this time examined ethnic differences induced by corticosteroid application (a vasoconstrictive stimulus).[9] They examined six black and eight Caucasian men, matched for age, and measured cutaneous hyperemia using LDV, before and after the application of 0.05% clobetasol ointment to the forearm. The following parameters were analyzed: (i) the area under the curve response from the starting point of the hyperemic response until the return of blood flow to basal values; (ii) the magnitude of the maximum peak response; (iii) the slope of the rise from immediate postocclusion to peak reactive hyperemic flow; and (iv) the slope of the decay from peak reactive hyperemic flow to resting levels. After the vasoconstrictive stimulus was given, the black subjects showed a 40% decreased area under the curve response ($p < 0.04$), a 50% decreased peak response ($p < 0.01$), and a decreased decay slope after peak blood flow ($p < 0.04$) compared with the whites. Overall, their data were consistent with a decrease in blood vessel reactivity of blacks compared with whites.

Gean et al.[28] also found differences in blood vessel reactivity between different ethnic groups; however, their data conflict with the findings of Berardesca and Maibach.[9] Gean et al.[28] examined five black subjects (skin types V or VI), five Asian subjects (skin type IV), and five Caucasian subjects (skin type II), aged 20–35 years, with no history of skin disease, who were nonsmokers and were not taking prescription medications, and applied three different concentrations of methyl nicotinate to the upper third of the ventral forearm. Methyl nicotinate-induced vasodilation was assessed visually and by LDV. At three different dose levels, the following parameters were compared: (i) the diameter of the maximum visually perceptible erythematous area; (ii) the area under the erythematous diameter versus time curve; (iii) the maximum LDV response; and (iv) the area under the LDV response versus time curve. Since we are reviewing only the objective data, the first two parameters measuring erythema revealed no significant differences and will not be discussed. The investigators observed that the area under the curve for LDV response versus time was greater in blacks than Caucasians for all methyl nicotinate concentrations ($p < 0.05$). This contrasts with prior studies, which found either no difference[7,31] or a decrease[9] in the area under the curve response in blacks. Note, however, that in this study a vasodilator (methyl nicotinate) was given, whereas in the prior study by Berardesca and Maibach[9] a vasoconstrictor was given. They also found that the area under the curve response versus time was greater in Asians compared with Caucasians for higher dose levels of methyl nicotinate ($p < 0.05$).

Kompaore et al.[39] evaluated TEWL and lag time to vasodilatation by LDV, before and after removal of the SC by tape stripping in seven black men, eight Caucasian subjects (six male and two female), and six Asian men. After application of methyl nicotinate, but before tape stripping, there was no difference between the groups in basal perfusion flow (by LDV), but lag time before vasodilatation was greater in blacks and less in Asians compared with Caucasians ($p < 0.05$). After 8 and 12 tape strips, lag time before vasodilatation decreased in all three groups, but decreased significantly more in Asians compared with Caucasians and blacks ($p < 0.05$). The order of sensitivity to methyl nicotinate was Asian > Caucasian > black. After topical application of methyl nicotinate, TEWL measurements indicated that black and Asian skin was more permeable to water than Caucasian skin (see Table 2.1); however, LDV-recorded lag time to vasodilatation results revealed that Asian skin had a higher permeability to methyl nicotinate than Caucasian and black skin. This study confirmed the importance of the SC in barrier function, but could not explain the reason behind the ethnic differences in TEWL and lag time to vasodilatation.

Aramaki et al.[3] evaluated LDV at baseline and after SLS-induced irritation in 22 Japanese and 22 German women. There was no difference in LDV at baseline and after SLS-induced irritation. Few studies measuring LDV have examined persons of Asian descent. Although it is difficult to compare a study that used tape stripping[39] with one that used a vasoactive substance,[3] note that Aramaki et al.[3] had a larger sample size than Kompaore et al.[39] and found no baseline difference in LDV.

An investigation done by Hicks et al.[33] demonstrated no significant difference in blood vessel reactivity between black and white participants. SLS was applied to the volar forearm, and response was recorded using LDV. The results obtained are in conflict with several previous studies that have suggested differences between black and white skin.[9,28,31,39] However, the investigators expressed doubt in the validity of the LDV measurements due to technical difficulties in using the flowmeter while conducting the study.

TABLE 2.4
Blood Vessel Reactivity[a]

Study	Technique	Subjects	Site	Results
Guy et al.[31]	Topically administered MN (vasodilator); LDV and PPG	Blacks 6 (age 20–30 y) Whites 6 (age 20–30 y) Whites 6 (age 63–80 y)	Volar forearm	**MN given** • No significant difference in time to peak response, area under response–time curve, or time for response to decay to 75% of its max value • PPG max response young black (40% less) < young white ($p < 0.05$)
Berardesca et al.[7]	Topically administered SLS (irritant); LDV	Black men 10 (age 29.9 ± 7.2 y) White men 9 (age 30.6 ± 8.8 y)	Back	**SLS stress** • No significant difference between blacks and whites • Blood vessel reactivity minimal in blacks from baseline to application of 0.5% SLS on untreated skin
Berardesca et al.[8]	Topically administered SLS (irritant); LDV	Hispanic men 7 (age 27.8 ± 4.5 y) White men 9 (age 30.6 ± 8.8y)	Upper back	**SLS stress** • Similar LDV response in Hispanics and whites
Berardesca et al.[9]	Topically administered corticoid (vasoconstrictor); LDV	Black men 6 Caucasian men 8 (mean age 27 ± 3 y, both)	Forearm	**After vasoconstrictor given** • 40% decreased area under the curve response blacks compared with whites ($p < 0.04$) • 50% decreased peak response in blacks compared with whites ($p < 0.01$) Decreased decay slope after peak blood flow in blacks compared to Caucasians; in blacks, $y = 3.3672–0.0737\times$ before treatment compared to $y = 2.5347–0.0367\times$ after treatment ($p < 0.04$), i.e., less blood vessel reactivity in blacks
Gean et al.[28]	Topically administered MN (vasodilator); LDV	Blacks 5 Caucasians 5 Asians 5 (age 20–35 y, all)	Upper 1/3 volar forearm	**MN given** • Area under the curve for LDV response versus time blacks > Caucasians for all MN concentrations ($p < 0.05$) • Area under the curve for LDV response versus time Asians > Caucasians for higher dose levels of MN ($p < 0.05$)
Kompaore et al.[39]	Topically administered MN (vasodilator); LDV	Blacks 7 Caucasians 8 Asians 6 (age 23–32 y, all)	Volar forearm	**MN given** • Before tape stripping: no difference between the groups in basal perfusion flow, but lag time before vasodilatation was blacks > Caucasians > Asians ($p < 0.05$) • After 8 and 12 tape strips: lag time before vasodilatation decreased in all three groups, but significantly decreased in Asians > Caucasians > blacks ($p < 0.05$)
Aramaki et al.[3]	Topically administered SLS (irritant); LDV	Japanese women 22 (mean age 25.84 y) German women 22 (mean age 26.94 y)	Forearm	• No significant difference at baseline or after SLS stress

TABLE 2.4 (continued)
Blood Vessel Reactivity[a]

Study	Technique	Subjects	Site	Results
Hicks et al.[33]	Topically administered SLS (irritant); LDV	White 7 Black 6 (age 18–40 y, all)	Volar forearm	• SLS stress: no significant difference in LDV response between groups

Note: Corticoid = clobetasol propionate 0.05% ointment; LDV = laser Doppler velocimetry; MN = methyl nicotinate; PPG = photoplethysmography; SLS = sodium lauryl sulfate; y = years.

[a] Studies cannot be compared to each other because each uses different vasoactive substances. However, each study, except for Berardesca and Maibach[8] comparing Hispanics and whites, Aramaki et al.[3] comparing Japanese and German women, and Hicks et al.[33] comparing blacks and whites, reveals some degree of ethnic variation in blood vessel reactivity.

Source: Wesley, N.O. and Maibach, H.I., *Am. J. Clin. Dermatol*., 4(12), 843–860, 2003. With permission.

The results of the studies on blood vessel reactivity are summarized in Table 2.4. Since each study administered different vasoactive substances that may act on different receptors on blood vessels, they could not be objectively compared.[36] As was noted by Hicks et al.,[33] it has been previously reported that small changes in position of the measuring probe can produce significant changes in measurements and may result in decreased reliability of results. Additionally, measurements may differ according to anatomic sites.

2.6 ELASTIC RECOVERY/EXTENSIBILITY

In addition to examining TEWL and skin conductance, Berardesca et al.[12] also examined biomechanical properties, such as elastic recovery and skin extensibility, on the dorsal and volar forearm in 15 blacks, 12 whites, and 12 Hispanics. These biomechanical properties were determined by applying a specific torque parallel to the skin's surface and then measuring how stretchable the skin was (skin extensibility) and recording the time required for the skin to return to its original state after release of the torque (elastic recovery). For skin elastic recovery, they found no significant difference between the races on the dorsal forearm (blacks > whites, but not significant). However, elastic recovery was 26% less in blacks compared with whites on the volar forearm ($p < 0.001$). There was no significant difference in elastic recovery between whites and Hispanics. The authors explained the significantly decreased elastic recovery in blacks compared with whites on the volar forearm, with a higher recovery in blacks on the dorsal side (although not significant), on the basis of greater actinic damage on the dorsal side of whites, with melanin as a photoprotective factor in blacks.

For skin extensibility, within each race, Berardesca et al.[12] found significant differences between dorsal and volar forearms in Hispanics and whites (dorsal < volar) ($p < 0.0002$ and $p < 0.0001$, respectively), but extensibility was the same on both sides of the forearm in blacks. When comparing the races to each other, blacks had greater extensibility than whites on the dorsal forearm, but decreased extensibility than whites on the volar forearm ($p < 0.01$ for both). Skin elasticity overall is defined as elastic recovery divided by extensibility. When looking at this ratio the investigators found no significant differences between the races. They explained the variability in these biomechanical properties of skin based on the protective role of melanin from UV rays. They believed that blacks did not show differences in skin extensibility between the dorsal and volar forearm because they were more photoprotected. Furthermore, they believed that blacks had greater extensibility on the dorsal forearm compared with whites for the same reason. However, if blacks are presumed to also be more photoprotected on the volar forearm compared with whites, this reasoning does not explain why whites were found to have a greater extensibility than blacks on the volar side.

Warrier et al.[83] examined elastic recovery in 30 black and 30 white women but did not record skin extensibility. There was no significant difference between blacks and whites on the legs, but elastic recovery on the cheeks was 1.5 times greater in blacks than in whites ($p < 0.05$). These findings contradicted those of Berardesca et al.[12] who found a 26% decrease in elastic recovery on the volar forearm of blacks. Warrier et al.[83] explained their findings of higher elastic recovery on the cheeks of blacks based on the higher WC that they found on the same anatomic area, thus presumably resulting in a higher elastic deformation.

The data on skin biomechanics, specifically elastic recovery and extensibility, vary by anatomic site and by race. However, the conclusions drawn by Berardesca et al.[12] contradict those of Warrier et al.[83] The data not only vary by race and by site, but age may also be a contributing factor. In the study by Berardesca et al.,[12] the subjects were all within the same age range (mean age 46.7–49.8 years). However, even though Warrier et al.[83] had a larger number of study subjects, the age range was 18–45 years. Overall, the ethnic differences in skin biomechanics are inconclusive and warrant further study (see Table 2.5).

2.7 MICROTOPOGRAPHY

Skin microrelief reflects the three-dimensional organization of the deeper layers and functional status of the skin.[30] Research has been performed relating changes in skin

TABLE 2.5[a]

Study	Technique	Subjects	Site	Results
(a) Skin Elastic Recovery				
Berardesca et al.[12]	*In vivo*	Blacks 15 (mean age 46.7 ± 2.4 y) Whites 12 (mean age 49.8 ± 2 y) Hispanics 12 (mean age 48.8 ± 2 y)	Volar and dorsal forearm	• No significant difference between groups on dorsal forearm • Elastic recovery Blacks (26% less) < whites on volar forearm ($p < 0.001$)
Warrier et al.[83]	*In vivo*	Black women 30 White women 30 (age 18–45 y, both)	Left and right medial cheeks, midvolar forearms, lateral midlower legs	• No significant difference between groups on the legs • Elastic recovery Blacks 1.5× > whites on cheeks ($p < 0.05$)
(b) Skin Extensibility				
Berardesca et al.[2]	*In vivo*	Blacks 15 (mean age 46.7 ± 2.4 y) Whites 12 (mean age 49.8 ± 2 y) Hispanics 12 (mean age 48.8 ± 2 y)	Volar and dorsal forearm	• Significant dorsal < volar extensibility within whites and Hispanics ($p < 0.001$ and $p < 0.002$, respectively) • Black > white extensibility dorsal forearm ($p < 0.01$) • Black < white extensibility volar forearm ($p < 0.01$)
(c) Microtopography				
Guehenneux et al.[30]	*In vivo*—skin replicas and interferometry	Caucasian 356 Japanese 120 (age 20–80 y, women, all)	Volar forearm	• ↑ in the density of lines > 60 μm and ↓ in the density of lines < 60 μm in depth with increasing age in both; change in Caucasians > Japanese and at earlier age in Caucasians • Anisotropy: ↑ with age in Caucasians, no change in Japanese
Diridollou et al.[21]	*In vivo*—SkinChip	310 women (age 18–61 y, all; African American, Caucasian, Asian, Hispanic)	Dorsal and ventral forearms	• Roughness and anisotropy ↑ with age on both dorsal and ventral forearms in all groups; Caucasians > Hispanic and Asians and African Americans • Density of the line intersections: Caucasians and Hispanics < Asians and African Americans
(d) pH Gradient				
Berardesca et al.[11]		Black women 8 Caucasian women 10 (mean age 42.3 ± 5 y, both)	Midvolar forearm	• No significant difference in pH at baseline **After tape stripping** • pH significantly decreased in blacks after three tape strips, i.e., superficial SC layers • No differences between ethnicitiess after 9, 12, and 15 tape strips, i.e., deeper SC layers
Warrier et al.[83]		Black women 30 White women 30 (age 18–45 y, both)	Left and right medial cheeks, midvolar forearms, lateral midlower legs	• pH blacks (pH = 5.15) < whites (pH = 5.52) on cheeks at baseline ($p < 0.05$) • No significant difference in pH on the legs at baseline
Grimes et al.[29]		African American 18 White 19 (age 35–65 y, women, all)	Above left eyebrow	• Baseline: African Americans < whites, but not statistically significant

TABLE 2.5 (continued)

Study	Technique	Subjects	Site	Results
(e) Lipid Content				
Reinertson and Wheatley[61]		*Cadavers*: Black man 1 White man 3 *Living*: Black man 1 White man 1 (age 49–68 y, all)	**Cadavers** Abdomen **Living** Back and thigh	• Lipid and sterol content in total epidermis Blacks > whites
Sugino et al.[70]		Black, white, Hispanic, and Asian (no. of subjects, age not specified)	Not documented	• Ceramide levels blacks (50% less) < whites and Hispanics ($p < 0.05$)
Harding et al.[32]		UK 41 Thai (dry season) 31 Thai (humid season) 31 (age 20–40 y, all)	Scalp	• UK and Thai subjects demonstrated similar levels of total lipids
(f) Sebaceous Function				
Aramaki et al.[3]	*In vivo*—sebumeter; topical application of SLS (irritant)	Japanese women 22 (mean age 25.84 y) German women 22 (mean age 26.94 y)	Forearm	• Baseline sebum levels: Japanese < whites ($p < 0.05$) • After SLS stress: Japanese > whites ($p < 0.05$)
Grimes et al.[29]	*In vivo*—sebumeter	African American 18 White 19 (age 35–65 y, women, all)	Forehead	• Baseline sebum levels: African Americans < whites, but not statistically significant
de Rigal et al.[19]	*In vivo*—sebumeter; sebutape	387 women (age 18–70 y, all; African American, Hispanic, Caucasian, Chinese)	Forehead and cheeks	• Mean sebum excretion rate: same across all ethnic groups • Number of sebaceous glands: Chinese and Hispanics < Caucasians and African Americans. • Sebum level decrease with age: linear in Chinese; sudden ↓ around age 50 y for other 3 groups
(g) Mast Cell Granules				
Sueki et al.[69]	EM of biopsy specimen	Black men 4 (mean age 29.2 ± 3 y) Caucasian men 4 (mean age 29.4 ± 1.2 y)	Medial-lateral buttock	• Mast cells contain 1.5× larger granules in black skin compared to white skin ($p < 0.0001$) • Mast cells contain 15% more PLS in Blacks compared to whites ($p < 0.05$) • Mast cells contain 30% less curved lamellae in blacks compared to whites ($p < 0.05$) • Tryptase immunoreactivity localized to PLS regions in black skin, compared to curved lamellae regions in white skin ($p < 0.0001$) • Cathepsin G localized to electron-dense amorphous subregions in both black and white skin

(*continued*)

TABLE 2.5 (continued)

Study	Technique	Subjects	Site	Results
(h) Vellus Hair Follicles				
Mangelsdorf et al.[44]	*In vivo*—skin surface biopsies	Asian 10 African American 10 (age 25–50 y, males, all) (results compared to Caucasians studied in Otberg et al.[52])	Forehead, back, thorax, upper arm, forearm, thigh, calf	• Distribution of follicle density for different body sites same in all groups: highest on forehead, lowest on calf. • Follicle density on forehead: Caucasians > African Americans > Asians ($p < 0.01$); no significant differences on other sites. • Calf and thigh: Asians and African Americans – smaller values for volume ($p < 0.01$, both), potential penetration surface ($p < 0.01$, both), follicular orifice ($p < 0.01$ and $p < 0.05$, respectively), and hair shaft diameter ($p < 0.01$, both)

Note: EM = electron microscopy; PLS = parallel-linear striations; SC = stratum corneum; y = years.

[a] In summary, we are unable to draw conclusions regarding ethnic differences in skin biomechanics (skin elastic recovery and extensibility) due to insufficient and conflicting evidence. It is difficult to compare microtopography studies due to different techniques. However, studies demonstrate an increase in anisotropy with age in Caucasians. Three skin pH studies demonstrate pH of black less than white skin. However, Berardesca et al.[11] demonstrate this difference after superficial tape stripping of the volar forearm, but not at baseline; while Warrier et al.[83] demonstrate the difference at baseline on the cheeks but not on the legs; and the results from Grimes et al.[29] did not reach statistical significance. Ethnic differences in lipid content and sebaceous function are inconclusive. Ethnic differences in sebaceous function are inconclusive. Larger mast cell granules, increased PLS, and increased tryptase localized to PLS in black compared to white skin. Lastly, we are unable to draw conclusions regarding ethnic differences in vellus hair follicle distribution and morphology due to insufficient evidence.

Source: Wesley, N.O. and Maibach, H.I., *Am. J. Clin. Dermatol.*, 4(12), 843–860, 2003. With permission.

microtopography to age and, more recently, relating changes to ethnic origin (see Table 2.5). Guehenneux et al.[30] (abstract only) studied changes in microrelief with age in 356 Caucasian and 120 Japanese women, aged 20–80 years, whose volar forearms were examined via skin replicas and analyzed by interferometry, simultaneously during winter in Paris and Sendai. Of the 12 "global parameters" and 13 "local parameters," the abstract reported the analysis of 3 local parameters: orientation of lines, depth of lines, and anisotropy index. Both Caucasian and Japanese women showed an increase in the density of lines measuring >60 μm in depth and a decrease in the density of lines measuring <60 μm with increasing age. However, this change was found to be more pronounced and occur at a younger age in Caucasian women. In addition, although no changes in orientation of lines with age were found in Japanese women, changes correlating with an increase in skin anisotropy with age were found in Caucasian women. Note that it is difficult to assess the reliability of comparing these results as the subjects were studied in two distinct geographical locations where environmental exposures may differ.

A study by Diridollou et al.[21] (abstract only) compared skin topography among 310 women, aged 18–61 years, including subjects of African American, Caucasian, Asian, and Hispanic descent; the ethnic distribution was not delineated in the abstract. Skin microrelief of the dorsal and ventral forearms was investigated according to age and ethnicity in terms of the density of line intersections in which a higher density of the intersection indicated smoother skin, and line orientation in which a smaller angle difference between the two main directions of the lines indicated higher anisotropy. The results were gathered using SkinChip, a device based on active capacitance imaging technology and image analysis software. On the ventral forearms, the data supported that the roughness and anisotropy of the skin increases with age in all four ethnic groups; the density of intersection decreased and angle between lines of different orientation became smaller. The same results were produced by the dorsal forearms, a sun-exposed site, but changes were significantly less pronounced for the African-American subjects, indicating a possible resistance to photoaging in this group. Overall, the density of the intersections was less for Caucasians and Hispanics than for Asians and African Americans. In addition, the anisotropy was higher for Caucasians than for Hispanics or Asians, and significantly higher than African Americans.

Diridollou et al.[21] concluded that roughness and anisotropy are more pronounced in Caucasian skin, than in Hispanic, Asian, and African-American skin. Guehenneux et al.[30] also found more pronounced changes of topography and higher anisotropy in Caucasian skin as compared to Asian skin, and at an earlier age. However, the results of both studies cannot be compared or integrated as they used different tools of investigation and different evaluation parameters.

2.8 pH GRADIENT

Ethnic differences in pH of the skin have also been explored. In addition to examining TEWL, Berardesca et al.[11] also examined differences in pH in 10 Caucasian (skin types I and II) and eight black African-American (skin type VI) women at baseline and after tape strippings. They found no significant differences between the two races in pH at baseline. After tape stripping, however, they found a significantly lower pH in blacks compared with whites after three tape strippings, but no significant differences after 9, 12, and 15 strippings. Thus, there was a lower pH in black skin compared with white skin in the superficial layers of the SC, but not in the deeper layers. The investigators stated that the data were difficult to explain. It was hypothesized that since the TEWL was also found to be increased after 3 and 6 tape strippings, the increased TEWL might allow for an increase in the hydrogen ion concentration in a normally hydrophobic SC. Of note, although the difference between the races in pH was not significant at deeper layers of the SC, the pH in both races did decrease with more tape strippings, but TEWL did not follow the same trend. Thus, an increase in TEWL does not fully explain the findings in pH.

Warrier et al.[83] also included pH in their study of 30 black and 30 white women; however, they only examined pH at baseline, not after tape stripping. There was a decreased pH on the cheeks of blacks compared with whites, pH = 5.15 versus pH = 5.52, respectively ($p < 0.05$). There was also a decreased pH in blacks on the legs, but the difference was not significant. The authors attributed the decreased pH in blacks to lactic acid and dicarboxylic amino acids in sweat secretions mixed with sebum, and evaporation of sweat causing acidity to increase[20] with the idea that there might be a higher number of sweat glands in blacks.[48]

Similar results were also produced in the study by Grimes et al.[29] The skin pH, measured above the left eyebrow in 18 African American and 19 white women, aged 35–65 years, was found to be lower in African Americans than whites, but the results did not reach statistical significance.

The skin pH has been found to be lower in blacks compared with whites in three different studies, but under different circumstances. Berardesca et al.[11] only demonstrate this difference in the superficial layers of the SC on the volar forearm, but not at baseline; while Warrier et al.[83] demonstrate the significant difference at baseline on the cheeks but not on the legs. Grimes et al. [29] demonstrated a difference on the forehead, but lacked statistical significance. Thus, it can be implied that may be some difference between whites and blacks in SC pH but the etiology of this finding and its confounders remain to be explored (see Table 2.5).

2.9 LIPID CONTENT

Skin lipids may play a role in modulating the relation between SC water content and TEWL, resulting in higher conductance values in blacks and Hispanics; greater intercellular cohesion with a normal TEWL could produce a higher WC.[10] Sugino et al.[70] correlated high WC values with high ceramide (lipid) levels and low TEWL. They studied WC (by impedance), TEWL, and ceramide levels in black, white, Hispanic, and Asian subjects. The number of subjects, age, and sample site were not documented in the published abstract. Ceramide levels were 50% lower in blacks compared with whites and Hispanics (total ceramides: 10.7 ± 4.7 μg/mg, 20.4 ± 8.1 μg/mg, 20.0 ± 4.3 μg/mg, respectively; $p < 0.05$). Though they noted that WC levels were highest in Asians, they did not document the ceramide levels of Asians (according to their hypothesis, Asians should have the highest ceramide levels). Thus, the correlation that was made between WC, TEWL, and ceramide levels was not fully exemplified.

The finding of low ceramide levels in blacks by Sugino et al.[70] is important, because several studies base their findings of increased WC in blacks[12,83] upon a 1959 study by Reinertson and Wheatley[61] which, in contrast to Sugino et al., showed higher total epidermis lipid and sterol content in blacks compared with whites. They took abdominal skin from four cadavers (one black man and three white men), and back and thigh skin from one black and one white man, all aged 49–68 years, and examined lipid and sterol content. Although they found that lipid and sterol levels were higher in blacks, they had a small sample size and compared skin from both deceased and living subjects at different anatomic sites.

Harding et al.[32] analyzed scalp SC lipid content in 41 UK, 31 Thai (dry season), and 31 Thai (humid season) subjects, aged 20–40 years, in an attempt to evaluate ethnic differences in dandruff. They observed that decreased levels of scalp SC free fatty acids, cholesterol, and ceramides were found in subjects with dandruff. However, the overall levels of scalp lipids were similar in the UK and Thai subjects.

Overall, it seems as though ethnic differences in skin lipid content are inconclusive because one study finds decreased lipids in blacks compared with whites,[70] another finds increased lipids in blacks,[61] and still another finds no difference between people from the UK and Thailand (see Table 2.5).[32]

2.10 SEBACEOUS FUNCTION

Sebum is a semisolid secreted onto the skin surface by glands attached to the hair follicle by a duct.[57] The functions of sebum include protection from friction, reduction of water loss, and protection from infection. Sebum levels have been confirmed to decline with age; however, there are few studies on the effect of race on baseline sebum secretion. Grimes et al.[29] used a sebumeter to measure sebum levels on the forehead as a component of baseline parameters in their study of 18 African-American and 19 white women, aged 35–65 years. The results showed lower levels of sebum on African-American skin than on white skin, but differences were not statistically significant.

A study by de Rigal et al.[19] (abstract only) investigated the skin sebaceous function of 387 women of African-American, Hispanic, Caucasian, or Chinese descents, aged 18–70 years. Measurements were performed using a sebumeter and sebutape on the forehead and cheeks to compare sebum excretion

rate and number of sebaceous glands according to ethnicity and age. The mean gland excretion was the same across ethnic groups. However, the number of sebaceous glands was lower in Chinese and Hispanic groups as compared to Caucasian and African American groups. In addition, the normal sebum decrease with age was different in each population; the decrease was linear in the Chinese group, but the other three groups exhibited a sudden decrease around age 50 years.

Aramaki et al.[3] assessed sebum secretion as a part of their study investigating skin reaction to SLS at concentrations of 0.25 and 0.5%. Before and after application of SLS to the forearms of each subject, sebum levels were determined by a sebumeter. The baseline sebum levels were lower in Japanese women than in white women. However, after application of SLS 0.25 and 0.5%, sebum levels were higher in the Japanese women ($p < 0.05$).

The latter two studies suggest that significant differences exist between sebum levels according to ethnicity. The de Rigal et al.[19] study found that although, the mean sebum excretion was the same across ethnic groups, the number of sebaceous glands and the normal sebum decrease with age varied between groups. This may indicate a difference in distribution of sebum independent of sebum levels among ethnic groups. Aramaki et al.[3] determined sebum levels to be lower in Japanese women as compared to white women at baseline, but Japanese women expressed an increase in sebum levels in response to irritant stress. This irritant response may represent a physiologic attempt to increase barrier defense. Further studies will be useful to elucidate whether differences in barrier defense between ethnic groups are based on varying baseline sebum levels or varying sebaceous response to physical stress (see Table 2.5).

2.11 MAST CELL GRANULES

Based on frequent clinical observations of pruritus and scratching in African Americans, Sueki et al.[69] evaluated differences in mast cells between black and white skin (see Table 2.5). They took 4-mm punch biopsies of normal buttock skin from four African-American males (mean age 29.2 $\pm$ 3.0 years) and four white males (mean age 29.4 $\pm$ 1.2 years) with no prior history of skin disease or atopy and processed the biopsies routinely for electron microscopy. Mast cells in black skin contained 1.5 times larger granules ($p < 0.0001$), 15% more parallel-linear striations (PLS) ($p < 0.05$) and 30% less curved lamellae ($p < 0.05$) compared with white skin. In addition, the investigators also examined the subgranular distribution of mast cell proteases, tryptase, and cathepsin G by immunoelectron microscopy. They found that tryptase immunoreactivity localized to PLS regions in black skin, compared with curved lamellae regions in white skin ($p < 0.0001$). In contrast, cathepsin G localized to electron-dense amorphous subregions in both black and white skin.

The investigators attributed the larger mast cell granules in black skin to possible increased fusion or division of the granules in blacks. However, they noted that the larger percentage of PLS and smaller percentage of curved lamellae in blacks was more difficult to explain. They hypothesized that it might be influenced by the mediator content, especially the amount of tryptase. From other studies suggesting the participation of mast cells in aberrant fibrosis in skin disorders such as keloid scars[18] and hypertrophic scars,[37] the investigators suggested the involvement of tryptase in these disorders. Keloid scarring is frequently observed in black individuals and blacks were found to have increased amounts of tryptase in this study compared with whites. Even though the study had a small sample size and only examined skin from one anatomic region, the researchers still found significant structural differences in mast cells between black and white skin. Further investigation of proinflammatory mediators should be done to corroborate these findings. This discovery should also prompt further electron microscopic evaluation of other cells involved in dermatologic disorders.

2.12 VELLUS HAIR FOLLICLES

As follicular morphology and distribution may affect penetration of topical medications and consequent treatment response, Mangelsdorf et al.[44] investigated vellus hair follicle size and distribution in Asians and African Americans as compared to whites. Skin surface biopsies were taken from seven body sites of 10 Asians and 10 African Americans, aged 25–50 years. The body sites were matched to locations described by Otberg et al. [52] in their study on Caucasians. In comparing the results of the three ethnic groups, the distribution of follicle density at different body sites was the same; the highest average density was on the forehead and the lowest on the calf for all groups. However, follicular density on the forehead was significantly lower in Asians and African Americans ($p < 0.001$). The Asians and African Americans also exhibited smaller values for volume ($p < 0.01$, both groups), potential pentration surface ($p < 0.01$, both groups), follicular orifice ($p < 0.01$ and $p < 0.05$, respectively), and hair shaft diameter ($p < 0.01$, both groups) on the thigh and calf regions. In addition, the follicular reservoir, as described by follicular volume, was generally higher in Caucasians. The authors concluded that the significant ethnic differences in follicle structure and pattern of distribution, especially in calf and forehead regions, emphasize the need for skin absorption experiments on different skin types to develop effective medications for prevention and treatment of skin disorders.

2.13 EPIDERMAL INNERVATION

While TEWL, WC, and blood vessel reactivity have been used as measures of irritancy, Reilly et al.[60] sought to explain ethnic differences in irritancy in terms of differences in skin innervation and nociceptor activity. They utilized confocal microscopy to examine epidermal innervation of the volar forearm pretreated with capsaicin in 20 European Caucasian, 8 Japanese-American, and 8 Chinese-American volunteers. However, no differences in innervation, including the biochemical properties of the nerve fibers, was found.

2.14 MELANOSOMES

Ethnic differences in number of melanocytes, number of melanosomes, and morphology of melanosomes has been of great interest in working toward the development of objective definitions of skin color (see Table 2.6). The biosynthesis of melanin, a cutaneous pigment, occurs in a melanosome, a metabolic unit within the melanocyte; melanosomes are then transported via melanocyte dendrites to adjacent keratinocytes.[75]

In 1969, Szabo et al.[17] examined five Caucasoids, six American Indians, three Mongoloids (from Japan and China), and seven Negroids to observe melanosome groupings using electron micrographs. The melanosomes in keratinocytes of Caucasoids and Mongoloids were found to be grouped together with a surrounding membrane. In contrast,

TABLE 2.6[a]

Study	Technique	Subjects	Site	Results
(a) Melanosomes				
Szabo et al.[71]	*In vivo*—EM	Caucasoid 5 American Indian 6 Mongoloid 3 Negroid 7 (age not reported)	Not reported	• Caucasoids and Mongoloids: grouped melanosomes • Negroids: longer and wider melanosomes, predominantly individually dispersed
Alaluf et al.[1]	*In vivo*—EM; alkali solubility of melanin	European 10 Chinese 8 Mexican 10 Indian 10 African 10	Dorsal forearm and volar upper arm	• Average melanosome size: dorsal forearm > volar upper arm, in all ethnic groups ($p < 0.001$); African > Indian > Mexican > Chinese > European • Melanosome size ~ total melanin content ($p < 0.0001$) • Light melanin fraction: African < (Mexican and Chinese) < Indian < European • Dark melanin fraction: African and Indian > (Mexican and Chinese) > European • Total amount of melanin: African and Indian > Mexican and Chinese and European ($p < 0.001$)
Thong et al.[78]	*In vivo*—EM	Chinese 15 (Skin type IV/V, age 10–73 y) Caucasian 3 (Skin type II, age 22–49 y) African American 3 (Skin type VI, age 18–52 y)	Volar forearm	• Proportion of individually distributed to clustered melanosomes: African Americans > Asians > Caucasians ($p < 0.05$) • Mean ± SD size of melanosomes distributed individually > clustered, in all ethnic groups. • Mean ± SD size of random melanosomes: African Americans > Asians > Caucasians ($p < 0.05$)
(b) Skin Surface Microflora				
Rebora et al.[58]		Black men 10 White men 10 (age 21–59 y, both)	Forearm	• *Candida albicans* blacks (150% greater) > whites ($p < 0.025$) • Aerobes blacks (650% greater) > whites ($p < 0.025$)
Warrier et al.[83]		Black women 30 White women 30 (age 18–45 y, both)	Left and right medial cheeks, midvolar forearms, lateral midlower legs	• Density of *Propionibacterium acnes* blacks > whites, but not statistically significant • No significant difference in aerobes

Note: EM = electron micrograph; SD = standard deviation; y = years.

[a] Darker skin has more individually dispersed melanosomes in comparison to lighter skin, and individually dispersed melanosomes tend to be larger in size than clustered melanosomes. Additionally, there is insufficient and conflicting evidence to draw conclusions regarding ethnic differences in skin microflora.

Source: Wesley, N.O. and Maibach, H.I., *Am. J. Clin. Dermatol.*, 4(12), 843–860, 2003. With permission.

the Negroid keratinocytes showed numerous melanosomes, longer and wider than in other racial groups, and mostly individually dispersed. Additionally, they observed an increase in melanosomes of keratinocytes of all races after irradiation, with grouping of melanosomes maintained in Caucasoids and Mongoloids. The authors concluded that individually dispersed melanosomes give a more uniform and dense color than the grouping found in fair skin.

In 1973, Konrad et al.[40] studied melanosome distribution patterns in hyperpigmented white skin alone and found that when comparing hyperpigmented lesions to control areas, there were no uniform differences in the distribution patterns of melanosomes. In addition, the degree of clinical hyperpigmentation was not associated with specific distribution patterns. However, they did note an important relationship between melanosome size and distribution: the percentage of melanosomes dispersed singly increased with increasing melanosome size. The authors also reported findings with experimental pigment donation showing that large melanosomes are taken up individually by keratinocytes and dispersed singly within their cytoplasm while small melanosomes are incorporated and maintained as aggregates. This data suggested melanosome size differences as the basis for skin color differences between black skin and white skin.

More recently, Thong et al.[78] quantified variation in melanosome size and distribution pattern in Asian, Caucasian, and African-American skin. The volar forearms of 15 Chinese (phototypes IV–V; aged 10–73 years), 3 Caucasians (phototype II; aged 22–49 years), and 3 African Americans (phototype VI, aged 18–52) were examined by electron microscopy of 4-mm punch biopsies. The proportions of individual and clustered melanosomes were compared for each ethnic group and showed statistically significant differences ($p < 0.05$). Melanosomes in Caucasian skin were distributed as 15.5% individual versus 84.5% clustered. Meanwhile, in African Americans, the melanosomes were distributed as 88.9% individual versus 11.1% clustered. The Asian melanosome distribution was intermediate between the latter two groups, as 62.6% individual versus 37.4% clustered. The investigators also determined the mean ± standard deviation (SD) size of melanosomes distributed individually to be larger in comparison to those distributed in clusters for each ethnic group. The mean ± SD of random melanosomes in each group differed as African-American skin showed significantly larger melanosome size than Caucasian skin, and Asian skin showed melanosome size as intermediate between the two other two groups. Thus, there was a trend of progressive increase in melanosome size when moving from Caucasian to African-American skin that corresponded with the progression from predominantly clustered to predominantly individual melanosome distribution. In addition, degradation patterns of melanosomes in the upper levels of epidermis varied by ethnic group. As keratinocytes became terminally differentiated and migrated to the SC, melanosomes were completely degraded and absent in the SC of light skin, while intact melanosomes could be seen in the SC of dark skin. Asian skin showed an intermediate pattern where few melanosomes remained in the corneocytes; interestingly, the remaining melanosomes were predominantly individual, indicating that clustered melanosomes may be degraded more efficiently during this process.

Alaluf et al.[1] examined the morphology, size, and melanin content of melanosomes on the volar upper arms and dorsal forearms of 10 European, 8 Chinese, 10 Mexican, 10 Indian, and 10 African subjects living in South Africa. Four-millimeter punch biopsies were analyzed based on electron micrographs of melanosomes and on alkali solubility of extracted melanin. The melanosome size of dorsal forearm (photoexposed) skin was observed as approximately 1.1 times larger than melanosome size of volar upper arm (photoprotected) skin ($p < 0.001$) when data were pooled from all ethnic groups; each ethnic group separately showed a similar trend, but lacked statistical significance. In addition, a progressive and statistically significant increase in average melanosome size was observed when moving from European (light) to African (dark) skin types. The melanosome size was directly correlated with total melanin content in the epidermis of all subjects ($p < 0.0001$). When comparing the epidermal melanin content among ethnic groups, the investigators found as downward trend in the amount of alkali soluble melanin (light-colored pheomelanin and DHICA-enriched eumelanin) in epidermis as the skin type became progressively darker; African skin contained the lowest amount ($p < 0.02$). Indian skin presented an exception to this trend with higher concentrations of light melanin fractions than both Mexican and Chinese skin ($p < 0.05$). However, both African and Indian skin showed about two times more of the alkali insoluble melanin (dark-colored DHI-enriched eumelanins) than the Mexican, Chinese, and European skin types ($p < 0.001$). Overall, the melanin composition showed a trend toward higher fractions of alkali-soluble melanins while moving from darker (African) skin to lighter (European) skin. In addition, African and Indian skin revealed the highest total amount of melanin ($p < 0.001$) and did not differ significantly from each other. There was no significant difference in total epidermal melanin between the remaining groups.

Despite the data showing differences in number and distribution of melanosomes, recent studies find no evidence of differences in numbers of melanocytes among ethnic groups.[75] For example, Alaluf et al.[2] found no significant difference in melanocyte number between African ($n = 10$), Indian ($n = 10$), Mexican ($n = 10$), or Chinese ($n = 8$) skin types using immunohistochemical methods. They did consistently find 60–80% more melanocytes in European ($n = 10$) skin than all other skin types ($p < 0.01$), but the authors felt a larger sample size would be necessary to confirm this observation. Tadokoro et al.[73] also found approximately equal densities of melanocytes in unirradiated skin of Asian, black, and white subjects ranging from 12.2 to 12.8 melanocytes per millimeter.

Thus, it is generally accepted that differences in skin color are supported more by differences in melanosome distribution, size, and content rather than melanocyte number. Szabo et al.[71] observed larger and more individually dispersed melanosomes in Negroid keratinocytes and

concluded that individually dispersed melanosomes may contribute to a more dense skin color. Konrad et al.[40] further noted that the number of singly dispersed melanosomes increased as melanosome size increased. Thong et al.[78] quantified the ethnic differences in melanosome size and distribution, finding a gradient in relative proportion of individual versus clustered melanosomes that corresponded with size of melanosomses. At one extreme, African-American skin showed larger melanosomes that were predominantly individually dispersed; and with Asian skin displaying intermediate results, Caucasian skin was at the other extreme, showing smaller melanosomes that were predominantly clustered. Alaluf et al.[1] also revealed a progressive increase in melanosome size as ethnic skin went from lighter to darker. Furthermore, dark skin contained more total melanin and a larger fraction of DHI-enriched (dark-colored) eumelanin than light skin.

2.15 SURFACE MICROFLORA

Ethnic differences in skin microflora have also been examined. Rebora and Guarrera[58] inoculated the forearm skin of 10 black men and 10 white men, aged 21–59 years, with *Candida albicans* and examined the severity of ensuing dermatitis as well as the population of *Candida* and other aerobes at the inoculum site. The severity of dermatitis was scored subjectively by observation of pustules. However, population of microflora was assessed objectively by colony counts after aerobic incubation at 95°F (35°C) for 2 days. Black skin harbored 150% more yeast after inoculation with *C. albicans* and 650% more aerobes both at baseline and after inoculation than white skin ($p < 0.025$).

In addition to investigating TEWL, capacitance, desquamation index, elastic recovery, and skin pH, Warrier et al.[83] also examined facial skin microflora in 30 black and 30 white women, aged 18–45 years. They found no significant differences in the density of aerobes (mostly *Staphylococcus* spp.) between blacks and whites. In contrast, although not statistically significant, there was a higher density of *Propionibacterium acnes* in blacks compared with whites. They felt that this might be due in part to a believed increase in sebum output in blacks.[38]

Both studies demonstrated increased skin microflora in blacks in that Rebora and Guarrera[58] found that blacks harbor significantly more *C. albicans* after inoculation, and Warrier et al.[83] found higher density of *P. acnes,* but the values were not statistically significant. However, Rebora and Guarrera[58] found blacks to have significantly higher levels of aerobes both at baseline and after inoculation with *C. albicans,* while Warrier et al.[83] found no significant ethnic differences in the density of aerobes. Since the minimal data that exist are conflicting, no conclusions regarding skin microflora can be made until investigators examine the issue further (see Table 2.6). Perhaps the age of subjects, anatomic site, and humidity of the geographic environment where the study was conducted cause variation in skin microflora and should be accounted for in the future studies.

2.16 ANTIMICROBIAL PROPERTIES

In 2001, Mackintosh[43] reviewed evidence discussing the role of melanization of skin in the innate immune defense system. He reported that a major function of melanocytes, melanosomes, and melanin in skin is to inhibit the proliferation of bacterial, fungal, and other parasitic infections in the dermis and epidermis. Numerous studies are cited showing evidence that melanocytes and melanosomes exhibit antimicrobial activity and are regulated by known mediators of inflammatory response. The review aims to support the hypothesis that immunity and melanization are genetically and functionally linked. The author notes that previous reports have implied a reduced susceptibility of dark-skinned individuals to skin disease. In addition, it is postulated that the evolution of black skin could represent high pressures from infection, especially in tropical regions. In five out of six recent investigations, people of African descent have been shown to be less susceptible than whites to scabies, fungal dermatophytosis, cutaneous *C. albicans* infections, and bacterial pyodermas. Additionally, although Rebora and Guarrera[58] demonstrated increased skin microflora in blacks, they found that the severity of dermatitis in black subjects was significantly less ($p < 0.01$) suggesting the possibility of increased barrier defense. This evidence may explain the existence of melanocytes and melanization among different parts of the body is independent of sun exposure, as in the genitalia, as well as the latitudinal gradient in skin melanization. The evolutionary data presented in this review article are compelling and indicate a necessity for controlled studies to clarify whether the number or melanocytes, size of melanosomes, or type of melanin can affect the antimicrobial properties of skin.

2.17 PHOTODAMAGE

Although there is evidence for objective differences in skin color, it remains unclear what role these differences in melanin and melanosomes play in dermatologic disorders. Section 2.16 of this chapter introduced the potential role of melanosomes in antimicrobial defense. The most extensively studied function of darker skin color, however, has been in resistance to photodamage from UV radiation. End effects of photodamage include skin cancer, which are well documented as affecting lighter skinned individuals more than those with darker skin.

In determining a relationship between melanosome groupings and sun exposure, studies have observed that dark-skinned whites, when exposed to sunlight, have nonaggregated melanosomes, in contrast to light-skin, unexposed whites who have aggregated melanosomes. Similarly, there are predominantly nonaggregated melanosomes in sunlight-exposed Asian skin, and primarily aggregated melanosomes in unexposed Asian skin.[62,75]

Alaluf et al.[1] noted an increase in melanosome size in photoexposed skin versus photoprotected skin in all ethnic groups; the melanosome size was directly correlated with epidermal melanin content, suggesting increased melanogenesis

in photoexposed areas. Van Nieuwpoort et al.[81] demonstrated that with increased melanogenesis, light skin melanosomes showed elongation and reduction in width with no significant change in surface area, while dark skin melanosomes enlarged in both length and width with an increase in volume. Based on these data, although all skin types show an increase in epidermal melanin with sun exposure, both distribution and morphology may influence unequal filtering between light and dark skin types.

In another study, Rijken et al. [63] investigated response to solar-simulating radiation (SSR) among white and black skin. Six healthy Dutch white subjects, with skin phototype I–III and mean age of 24.5 years, were exposed to 12,000–18,000 mJ/cm^2 of SSR. Six healthy West-African or Afro (South) American black subjects, skin phototype VI, and mean age of 25.3 years, were exposed to 18,000 mJ/cm^2 of SSR. Six other white subjects were also added to study the effects of erythema effective doses of SSR. Skin pigment, DNA photodamage, infiltrating neutrophils, photoaging-associated proteolytic enzymes, keratinocyte activation, and the source of interleukin 10 (IL-10) in skin biopsies taken before and after radiation. The significance of IL-10 lies in the fact that IL-10 producing cells may be involved in skin carcinogenesis. In each white volunteer, SSR caused DNA damage in epidermal and dermal cells, an influx of neutrophils, active proteoytic enzymes, and keratinocyte activation. Also, three white volunteers showed IL-10 producing neutrophils in the epidermis. In black-skinned individuals, aside from DNA damage in the suprabasal epidermis, there were no other changes found; basal keratinocytes and dermal cells were not damaged. The authors concluded that these results were best explained by difference in skin pigmentation and that melanin functions as a barrier to protect basal keratinocytes and the dermis from photodamage.

Other studies have suggested that filter properties of melanin alone do not provide sufficient protection against DNA damage in underlying cells. Tadokoro et al.[72] investigated the relationship between melanin and DNA damage after UV exposure in 37 subjects of 5 ethnic origins (black, white, Asian, others not specified), and Fitzpatrick phototypes I through VI. They found measurable damage to DNA in all groups, and DNA damage was maximal immediately after irradiation, gradually returning to baseline over time. The immediate DNA damage levels were higher in whites and Asians in comparison to blacks and Hispanics. In addition, the whites and Asians showed lower constitutive levels of melanin content. However, the kinetics of DNA damage differed among subjects. Upon monitoring the percentage of removal of damage toward baseline 7 days after UV exposure, no correlation was found between melanin content or ethnic group and the efficiency of DNA damage removal. There were variable rates of DNA repair within individual groups indicating that DNA repair rates were not associated with skin type. The authors noted that other properties of melanin, such as antioxidant properties and radical scavenging properties, may play roles in minimizing UV damage. Ethnic differences in expression of receptors involved in melanosome uptake and melanocyte-specific proteins, both before and after UV exposure, are also being investigated.

2.18 CONCLUSION

The U.S. census bureau estimates that the population is composed of 12.1% black or African American, 13.9% Hispanic, or Latino, and 11.9% other nonwhites.[80] It has been predicted that people with skin of color will constitute a majority of the United States and international populations in the twenty-first century.[56] These statistics highlight the importance of objective investigation of differences in structure and function of skin of different colors, relationships between race, color, ethnicity, and process and presentation of disease in these groups. It is imperative that we have a deeper understanding of these characteristics, as they play an important role in learning how to appropriately modify patient treatment. Differences do exist in structure and physiology of skin among different races, and may differentially affect disease. However, data on ethnic differences in skin, physiology, and function are few; the studies that do exist consist of typically small patient populations. Consequently, few definitive conclusions can be made. The FDA currently recommends inclusion of more ethnic groups in dermatologic trials, citing evidence that physiologic differences in skin structure between races can result in varying efficacies of dermatologic and topical treatments.[15]

There exists reasonable evidence (Table 2.7) to support that black skin has a higher TEWL, variable blood vessel reactivity, lower skin surface pH, larger mast cell granules, and larger melanosomes with more individual distribution when compared with white skin by means of objective measurements. Although some deductions have been made about Asian and Hispanic skin, the results are contradictory and further evaluation of Asian and Hispanic skin needs to be done. A review by Robinson[64] also supported the notion that the evidence comparing Asian and Caucasians is insufficient and less than compelling. Perhaps more specificity about the origin of their heritage should also be included since "Asian" and "Hispanic" encompass a broad spectrum of people. Ethnic differences in skin WC, corneocyte desquamation, skin elastic recovery/extensibility, microtopography, lipid content, sebaceous function, follicular morphology and distribution, and skin microflora, although statistically significant, are minimal and contradictory. Thus, no conclusions regarding these objective data can be made.

One issue that must be raised when interpreting these studies is the definition of race or ethnicity. Race seems to emcompass genetic variations based on natural selection, which include, but are not limited to pigmentation,[15] pigmentation appears to be based mainly on erythema, melanin, and the skin's response to physiologic insult. Anthropologists divide racial groups into Caucasoid (e.g., Europeans, Arabs, Indians), Mongoloid (e.g., Asians), Australoid (e.g., Australian

TABLE 2.7
Summary

Evidence Supports	Insufficient Evidence for	Inconclusive
• TEWL black > white skin • Variable ethnic blood vessel reactivity • pH black < white skin • Larger mast cell granules, increased PLS, and increased tryptase localized to PLS in black compared to white skin • Darker skin has more individually dispersed melanosomes; larger than clustered melanosomes	• Deductions regarding Asian and Hispanic skin Ethnic differences in:[a] • Skin elastic recovery/extensibility • Skin microflora • Epidermal innervation • Microtopography • Vellus hair follicle morphology/distribution	Ethnic differences in: • Water content • Corneocyte desquamation • Lipid content • Sebaceous function

Note: PLS = parallel-linear striations; TEWL = transepidermal water loss.

[a] Skin elastic recovery/extensibility, skin microflora, epidermal innervation, microtopography, and vellus hair follicle morphology/distribution were labeled as "insufficient evidence for" ethnic differences rather than "inconclusive" because only two studies or less examined these variables.

Source: Wesley, N.O. and Maibach, H.I., *Am. J. Clin. Dermatol.*, 4(12), 843–860, 2003. With permission.

aborigines), Congoid or Negroid (e.g., most African tribes and descendants) and Capoid (e.g., the Kung San African tribe) with the idea that racial variations were selected to facilitate adaptations to a particular environment.[16,76] Some reject the relevence of any genetic basis for race, stating that 90–95% of genetic variation occurs within geographic populations rather than across racial groups.[15] Furthermore, the concept of race has been dismissed by some as being an artificial, nongenetically defined construct, lacking scientific basis, and a hindrance for research, diagnosis, and treatment of skin disease.[15,86]

Ethnicity, in contrast, is a more general term, encompassing biologic and cultural factors. Ethnicity has been defined as how one sees oneself and how one is seen by others as part of a group on the basis of presumed ancestry and sharing a common destiny, often with commonalities in skin color, religion, language, customs, ancestry, and occupation or region.[51] Thus, ethnicity not only encompasses a set of categories that overlaps with race but also depends on more subjective and cultural factors, while race seems to encompass genetic variations based on natural selection. With these obscure definitions based on both biology and the subjective manner in which one labels oneself, the basis of objective research on racial or ethnic differences is already somewhat subjective and therefore problematic. However, studies show that differences, whether based on genetic variations or on subjective labels, do exist. Perhaps future studies in dermatology should also address how one defines oneself as part of a particular race or ethnic group in addition to examining degree of skin pigmentation. This will help determine whether the differences are truly the result of genetic variations that were selected for by race or of biologic variations in melanin content that vary between and within each race. Further research in both genetics and dermatology are warranted to draw any final conclusions with regards to race/ethnicity as the etiology for differences in skin physiology.

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3 Occlusion and Barrier Function

Hongbo Zhai and Howard I. Maibach

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3.1 INTRODUCTION

In general, occlusion may increase percutaneous absorption of applied compounds, but with exception (Bucks et al. 1988; Bucks et al. 1991; Bucks and Maibach 1999). It has been used in dermatology to increase topical corticosteroids efficacy (Scholtz 1961; Sulzberger and Witten 1961). However, it also obstructs normal ventilation of the skin surface; increases stratum corneum (SC) hydration, and hence may compromise skin barrier function (Agner and Serup 1993; Kligman 1996; Warner et al. 1999; Kligman 2000). Evaluation and investigation of the impact of occlusion on barrier function are important in skin physiology, pathology, pharmacology, and dermatology (Zhai and Maibach 2002). This updated chapter from Zhai and Maibach (2004) emphasizes the effects of occlusion on skin barrier function, particularly, as defined with objective skin bioengineering technology.

3.2 OCCLUSION AND ITS APPLICATION

With occlusion, the skin is covered directly or indirectly by impermeable films or substances such as diapers, tape, chambers, gloves, textiles, garments, wound dressings, transdermal devices, etc. (Kligman 1996). In addition, certain topical vehicles that contain fats or polymers oils (petrolatum, paraffin, etc.) may also generate occlusive effects (Berardesca and Maibach 1988).

Owing to its simplicity, occlusion is widely utilized to enhance the penetration of applied drugs in clinical practice. However, occlusion does not increase percutaneous absorption to all chemicals (Bucks et al. 1988; Bucks et al. 1991; Bucks and Maibach 1999). It may increase penetration of lipid-soluble, nonpolar molecules but has less effect on polar molecules: a trend of occlusion-induced absorption enhancement with increasing penetrant lipophilicity is apparent (Bucks et al. 1988; Treffel et al. 1992; Cross and Roberts 2000). In practice, increasing skin penetration rates of applied drug is far from simple. Skin barrier function can be ascribed to the macroscopic structure of the SC, consisting of alternating lipoidal and hydrophylic regions. For this reason, physico-chemical characteristics of the chemical, such as partition coefficient, structure, and molecular weight, play an important role in determining the facility of absorption (Wiechers 1989; Hostynek et al. 1996). Another factor to consider in drug percutaneous absorption is that the vehicle in which the drug is formulated acts on drug release from the formulation (Hotchkiss et al. 1992; Cross and Roberts 2000). Smith and Maibach (2005) provide an extensive overview of percutaneous penetration enhancers. In addition, the anatomical site may also influence the effects of occlusion on percutaneous absorption (Qiao et al. 1993).

In many industrial and food fields, protective gloves or clothing may protect the workers from hazardous materials or for hygiene. However, these protective measures may also produce negative events because of the nature of occlusion, which often causes SC hyperhydration and reduces the protective barrier properties of the skin (Graves et al. 1995). Many gloves do not resist the penetration of low molecular weight chemicals. As a result, those chemicals may enter the glove and become trapped on the skin under occlusion for many hours, possibly leading to irritation, and more seriously to dermatitis or eczematous changes (Van der Valk and Maibach 1989; Mathias 1990; Estlander et al. 1996; Chew and Maibach 2005).

Wound dressings have been employed to speed the healing processes in acute and chronic wounds. They keep

Modified from *Dermatotoxicology*, 6th Edition.

healing tissues moist and increase superficial wound epithelialization (Winter 1962; Winter and Scales 1963; Hinman and Maibach 1963; Alvarez et al. 1983; Eaglstein 1984; Berardesca and Maibach 1988). However, occlusive or semiocclusive dressings can increase microorganisms and hence induce wound infections (Aly et al. 1978; Rajka et al. 1981; Faergemann et al. 1983; Mertz and Eaglstein 1984; Berardesca and Maibach 1988). A significant increase in the density of *Staphylococcus aureus* and lipophilic diphtheroids were observed after 24 h occlusion in eczematous and psoriatic skin (Rajka et al. 1981).

Thus, the effects of occlusion on skin are complex and may produce profound changes that include altering epidermal lipids, DNA synthesis, epidermal turnover, pH, epidermal morphology, sweat glands, Langerhans cells stresses, etc (Faergemann et al. 1983; Berardesca and Maibach 1988; Bucks et al. 1991; Matsumura et al. 1995; Kligman 1996; Berardesca and Maibach 1996; Leow and Maibach 1997; Denda et al. 1998; Bucks and Maibach 1999; Warner et al. 1999; Kömüves et al. 1999; Fluhr et al. 1999; Kligman 2000).

3.3 SKIN BARRIER FUNCTION

Skin has numerous functions, one of which is to serve as a water permeability barrier to keep body fluids in and minimize dehydration. This function takes place largely in the SC or horny layer (Baker 1972). SC has been referred to as a brick and mortar structure. The bricks are protein-rich corneocytes separated by lipid-rich intercellular domains consisting of stacks of bilaminar membrane (Kligman 2000). Normally, the passage of water through the skin is closely controlled—allowing 0.5 cm^2/h to evaporate. When water content falls too low, water barrier function is impaired and the skin becomes more sensitive to repeated use of water, detergents, and other irritants. Barrier function may be disturbed by physical, chemical, pathological factors, and environmental changes (Denda et al. 1998).

Maintenance of the SC structural integrity is critical to barrier function. Increasing SC hydration can progressively reduce barrier efficiency (Bucks et al. 1991; Matsumura et al. 1995; Berardesca and Maibach 1996; Kligman 1996; Leow and Maibach 1997; Bucks et al. 1988; Haftek et al. 1998; Warner et al. 1999; Bucks and Maibach 1999; Fluhr et al. 1999; Tsai and Maibach 1999; Kligman 2000) and the changes of protein dynamics (Alonso et al. 2003). SC is extremely hygroscopic: it can pick up 500% of its dry weight in less than 1 h following immersing in water, swelling vertically to four to five times its original width (Kligman 2000).

3.4 EFFECTS OF OCCLUSION ON BARRIER FUNCTION

Healthy SC typically has a water content of 10–20% (Baker 1972). Occlusion can block diffusional water loss from skin surface, increasing SC hydration, thereby swelling the corneocytes, and promoting water uptake into intercellular lipid domains (Bucks et al. 1991; Bucks and Maibach 1999). Water content can be increased up to 50% with occlusion (Bucks et al. 1991; Bucks and Maibach 1999): even short-time (30 min) exposure can result in significantly increased SC hydration (Ryatt et al. 1988). With 24 h occlusion, the relative water content in SC can be increased significantly from 53% before occlusion to 59% (Faergemann et al. 1983). Occlusion of 24 h can induce morphological changes on the surface deepening skin furrows (Matsumura et al. 1995). Zhai et al. (2002) determined the level of skin hydration and skin permeability to nicotinates following occlusive patches and diapers at different exposure times. They found that permeation of nicotinates was increased for hydrated skin versus control even after only 10 min of patch exposure. No evidence of increased permeation rates with increased hyperhydration once a relatively low threshold of hyperhydration was achieved (e.g., which reached after a 10 min wet patch). Water under occlusion may disrupt barrier lipids and damage SC similar to surfactants (Warner et al. 1999). Kligman (1996) studied hydration dermatitis in man: 1 week of an impermeable plastic film did not injure skin; 2 weeks was moderately harmful to some but not all subjects; 3 weeks regularly induced dermatitis. Hydration dermatitis was independent of race, sex, and age. They examined the potential role of microorganisms in developing hydration dermatitis by using antibiotic solutions immediately following occlusion with plastic wrapping: microorganisms had no impact. In addition, hydrogels did not appreciably hydrate or macerate the surface by visual inspection when left in place for 1 week.

Some transdermal drug delivery systems (TDDS) may indeed provoke a dermatitis when applied twice weekly to the same site. These occlusive devices demonstrated marked cytotoxicity to Langerhans cells, melanocytes, and keratinocytes (Kligman 1996). However, Nieboer et al. (1987) evaluated the effects of occlusion with transdermal therapeutic systems (TTS) on Langerhans cell and skin irritation at different times ranging (6 h; 1, 2, 4, and 7 days). Irritation was judged on morphology, histopathologic, and immunofluorescence findings, and changes in the Langerhans cell systems. Occlusion provoked only slight or no skin irritation.

Bouwstra et al. (2003) recently reported that water only slightly changes the lipid transitions in the SC even at a hydration level of 300% wt/wt. No gradual increase in water level was observed in depth. At a very high hydration level (300% wt/wt), the corneocytes are strongly swollen except for the deepest cell layers adjacent to the viable epidermis. The corneocytes in these layers are not swollen. At 300% wt/wt hydration level, water domains are also present in intercellular regions. Between 17% wt/wt and 300% wt/wt, the cell thickness increases linearly with the hydration level suggesting that swelling of cells mainly occurs in the direction perpendicular to the skin surface. Fluhr et al. (1999) evaluated the barrier damage by prolonged occlusion on the forearm for 24–96 h and did not find significant changes in hydration and water-holding capacity. But transepidermal

water loss (TEWL) increased reaching a plateau on day two, concluding that occlusion induced barrier damage without skin dryness.

3.5 QUANTIFICATION WITH BIOENGINEERING TECHNIQUES

Recently, noninvasive bioengineering techniques have been utilized to better quantify skin barrier function. Modern noninvasive techniques can assess the mechanical and physiological properties of skin in health and disease. Their great value becomes apparent in providing accurate, reproducible, and objective measurements that can determine subtle differences before visual clinical signs. The unaided eye is not reliable for determining subclinical changes.

We briefly introduce some common useful noninvasive bioengineering techniques. Background, principles, extensive details, and validations of these techniques can be found in textbooks (Frosch and Kligman 1993; Elsner et al. 1994; Serup and Jemec 1995; Berardesca et al. 1995a,b; Wilhelm et al. 1997).

1. TEWL, as a marker of barrier function and structure changes, can be monitored by an evaporimeter (Tewameter) (Courage + Khazaka, Cologne, Germany, and Acaderm Inc., Menlo Park, California). It may also act as an indicator for the recovery of barrier function (Grubauer et al. 1989). Standard guidelines are utilized (Pinnagoda et al. 1990).
2. Cutaneous blood flux of test sites can be observed with a laser Doppler flowmeter (LDF) (Moor Instruments, Axminster, England) or laser Doppler velocimeter (LDV) (Acaderm Inc., Menlo Park, California). Methods and standard guidelines are described elsewhere (Bircher et al. 1994).
3. Skin color can be measured by a reflectance meter (such as a colorimeter) (Minolta, Osaka, Japan, and Acaderm Inc., Menlo Park, California), and the a* value (red–green axis) is considered a reliable quantification of erythema (Wilhelm et al. 1989; Wilhelm and Maibach 1989). Standard guidelines and measuring principle have been described in detail (Fullerton et al. 1996).
4. Capacitance as a parameter of SC hydration (or water content) can be determined with a capacitance meter (such as a Corneometer) (Courage + Khazaka, Cologne, Germany, and Acaderm Inc., Menlo Park, California). The measuring principle and methods are elsewhere (Triebskorn and Gloor 1993).

Brief quantification data of bioengineering measurements on occlusive skin conditions are summarized in Table 3.1.

TABLE 3.1
Brief Results of Bioengineering Measurements on Occlusive Skin Condition

Occlusive Manner and Time	Bioengineering Techniques	Results	References
Plastic film for 5 days	TEWL	TEWL increased from 0.56 mg/cm²/h (baseline) to 1.87 mg/cm²/h (occlusion) and showed essentially saturated after 2 days	Aly et al. (1978)
Plastic film for 1 h	TEWL and capacitance	Postocclusion TEWL was significantly greater than the normal sites	Orsmark et al. (1980)
Plastic film for 1, 3, and 8 days	TEWL and electromagnetic wave	Occlusion significantly increased TEWL and water content within 24 h	Faergemann et al. (1983)
Occlusion with polypropylene chamber or vehicles for 30 min; hexyl nicotinate (HN) as an indicator	LDV	The onset of action and time to peak were significantly shortened, and the peak height and area under curve (AUC) significantly increased under occlusion conditions	Ryatt et al. (1986)
Polypropylene chambers for 30 min; HN as an indicator	LDV	Occlusion significantly shortened both the time of onset of the LDV-detected response to HN and the time to peak response. In addition, the magnitude of the peak LDV response to HN and the AUC were significantly increased. Occlusion also significantly elevated the stratum corneum water content. There was a significant correlation between stratum corneum water content and area under the LDV response-time curve after 30 min occlusion	Ryatt et al. (1988)
Postapplication occlusion after short-term sodium lauryl sulphate (SLS) exposure for 5 consecutive days	TEWL	Occluded skin sites had a significant increase TEWL values (everyday and alternate-day schedule) when compared to unoccluded sites. Results indicated that postexposure occlusive treatment markedly enhanced irritant response	Van der Valk and Maibach (1989)
Chambers with 0.5% SLS, water, and empty chambers only for 3 h	TEWL	All values from SLS, water, and empty chambers were significantly increased as compared to normal skin	Pinnagoda et al. (1990)

(*continued*)

TABLE 3.1 (continued)
Brief Results of Bioengineering Measurements on Occlusive Skin Condition

Occlusive Manner and Time	Bioengineering Techniques	Results	References
Aluminum chambers and chambers with water, physiological saline, a paper disc or 0.002% of SLS for 24 h	Electrical impedance	Occlusion did not affect readings of electrical skin impedance taken 24 h or later after removal, but increased variance for readings taken 1 h after removal	Emtestam and Ollmar (1993)
Glove patch for 4 and 8 h, and empty dressing; HN as an indicator	LDV, TEWL, and skin surface roughness	Precorneal permeability, TEWL, compliance parameter were significantly increased after occlusion 4 and 8 h, and skin surface roughness was significantly reduced in terms of roughness parameters Ra and Rz by 4 and 8 h occlusion	Graves et al. (1995)
Plastic chambers with a series of sodium alkyl sulfates for 24 h	TEWL, capacitance, and a* values	All alkyl sulfates with the exception of sodium lauryl sulfate resulted in a temporary decrease of SC hydration 1 h after patch removal. At day 2, SC hydration levels of surfactant treated skin were not significantly different from controls. Thereafter, a second decrease in capacitance value was observed with lowest hydration at day 7	Wilhelm (1995)
Different occlusive and semipermeable dressings for 23 and 46 h on irritation and tape stripping skins	TEWL and capacitance	Occlusion did not significantly delay barrier repair	Welzel et al. (1995, 1996)
Short-term (6 h/day for 3 days) gloves on normal skin and gloves on SLS-compromised skin	TEWL, capacitance, and erythema index	Glove occlusion on normal skin for short-term exposure did not significantly change the water barrier function but caused a significantly negative effect on SLS-compromised skin for the same period	Ramsing and Agner (1996a)
Long-term (6 h/day for 14 days) gloves on normal skin and a cotton glove worn under the occlusive glove	TEWL, capacitance, and erythema index	This long-term using glove occlusion on normal skin caused a significant negative effect on skin barrier function, as measured by TEWL, which was prevented by the cotton glove	Ramsing and Agner (1996b)
Plastic chambers at 24, 48, 72, and 96 h	TEWL and capacitance	A significantly progressive increase under occlusion and reaching a plateau on day 2. Hydration and water-holding capacity did not show significant changes	Fluhr et al. (1999)
Occlusion with patches and diapers at different exposure times. Nicotinates as markers to evaluate skin permeability	Water evaporation rate (WER), skin blood flow volume (BFV), capacitance, and redness (a*)	Permeation of nicotinates was increased for hydrated skin versus control even after only 10 min of patch exposure. No evidence of increased permeation rates with increased hyperhydration once a relatively low threshold of hyperhydration was achieved (e.g., which reached after a 10 min wet patch)	Zhai et al. (2002)
Impermeable polyester film for 6 h	TEWL, impedance spectroscopy (IS) and attenuated-total-reflectance Fourier transform infrared (ATR-FTIR) spectroscopy	Occlusion of the skin either in the presence or absence of the cream caused TEWL to be increased when the treatment was terminated at 6 h. Uptake of ointment into the SC, on the other hand, inhibited the postapplication TEWL rate. In parallel, treatment with the ointment caused an increase in relatively low-frequency skin impedance, consistent with the entry of additional lipophilic constituents into the SC. The latter was confirmed by ATR-FTIR spectroscopic measurements	Curdy et al. (2004)

3.6 CONCLUSIONS

The effects of occlusion on skin barrier function have been defined with various techniques. Obviously, occlusion alone may damage skin barrier function. With application of chemicals/drugs under occlusion conditions, it can increase penetration of chemicals and antigens into the skin and therefore also increases dermatitis (Berardesca and Maibach 1988; Kligman 1996). Local reactions (i.e., irritation and sensitization) of TDDS, which are typically occlusive patches placed on the skin surface for 1–7 days to deliver the drugs into the systemic circulation have been widely reported (Boddé et al. 1989; Hogan and Maibach 1990, 1991; Patil et al. 1996; Murphy and Carmichael 2000). However, reactions can be minimized with immunosuppressive agents, antioxidants, local anesthetics, and other antiirritant technologies (Kydonieus and Wille 2000). Topical corticoids are another alternative but their role in the suppression of TDDS-induced dermatitis needs

better definition, especially for patients who require continued treatment with long-term application of such devices (Hogan and Maibach 1990). Advancements in design and construction of protective garments and wound dressings may reduce the level of skin hydration and dermatitis. Application of optimal hydrocolloid patches that absorb water in both liquid and vapor form can also decrease irritant reactions (Hurkmans et al. 1985; Fairbrother et al. 1992; Hollingsbee et al. 1995). A natural, pure, and nonwoven dressing has been made from calcium alginate fibers (Williams 1999). It can rapidly absorb and retain wound fluid to form an integral gellified structure, thereby maintaining an ideal moist wound-healing environment. It can also trap and immobilize pathogenic bacteria in the network of gellified fibers, stimulate macrophage activity and activate platelets, resulting in haemostasis and accelerated wound healing. Recent study indicated that with the silver-based wound dressing providing a more effective antimicrobial activity in a moist healing environment (Schaller et al. 2004).

Today, with the rapid development of the new technologies in the bioscience, we expect greater efficacy and optimal dressings or materials that can absorb excess water and reduce the unfavorable effects of occlusion.

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4 Anatomical Factors Affecting Barrier Function

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4.1 INTRODUCTION

Dermatotoxicology is the branch of science dealing with the assessment of responses of the skin to specific toxicants. It is thought that the primary function of skin is a barrier between the well-regulated "milieu interieur" and the outside environment. This may give one the impression that the structure of skin is simple and solely focused on its barrier properties. Past research in percutaneous absorption and dermatotoxicology has reinforced this view. However, more recent research in percutaneous absorption and dermal toxicology now take into consideration the possibility that additional anatomical factors may also affect the barrier function of skin, thereby altering the rate of absorption. Many earlier model systems used to evaluate percutaneous absorption were primitive and not capable of modeling all of these factors. Therefore, it is the purpose of this chapter to illustrate to scientists working in this field the complexity of the integument and how anatomical structures within the skin contribute to and influence its barrier function.

4.2 GENERAL CHARACTERISTICS

Skin is a complex, integrated, dynamic organ that has many functions (Table 4.1), which go far beyond its role as a barrier to the environment. Although metabolism and drug biotransformation are important, the reader is directed to the chapters on this subject in the present text. Skin (derived from the Latin meaning *roof*) is the largest organ of the body and is anatomically divided into the epidermis, which is the outermost layer, and the underlying dermis (Figure 4.1). The epidermis consists of a stratified squamous keratinized epithelium derived from ectoderm in which 80% of the cells are keratinocytes. Other cell types such as the melanocytes (pigment formation), Langerhans cells (immunological function), and Merkel cells (sensory perception) represent the nonkeratinocytes. The epidermis undergoes an orderly pattern of proliferation, differentiation, and keratinization. However, these processes are not fully understood. In addition, the epidermis can become specialized to form skin appendages such as hair, sebaceous and sweat glands, feathers, horn, digital organs (hoof, claw, nail), and specialized glandular structures.

The human epidermis consists of four to five cell layers depending on the body site. The first layer, the stratum basale, is a single layer of cuboidal- to columnar-shaped cells that are attached laterally to adjacent cells by desmosomes and to the irregular basement membrane by hemidesmosomes. The basal cell population is heterogeneous in that there are two morphologically distinct types. The first can function as stem cells and has the ability to divide and produce new cells, whereas the second serves to anchor the epidermis to the basement membrane (Lavker and Sun, 1982, 1983). The second outer layer is the stratum spinosum or "prickle cell layer," which consists of several layers of irregular polyhedral cells. These cells are connected to the stratum basale cells below and to the adjacent spinosum cells by desmosomes. The most prominent feature in this layer is the tonofilaments. Along with desmosomes, tight junctions (zona occludens) may connect cells to one another. It is in the uppermost layers of the stratum spinosum, that membrane coating or lamellar granules first appear. The third layer is the stratum granulosum that consists of several layers of flattened cells lying parallel to the epidermal–dermal junction. Irregularly shaped,

TABLE 4.1
Functions of Skin

Environmental barrier
Diffusion barrier
Metabolic barrier
Temperature regulation
Regulation of blood flow
Hair and Fur
Sweating
Immunological affector and effector axis
Mechanical support
Neurosensory reception
Endocrine
Apocrine/eccrine/sebaceous glandular secretion
Metabolism
Keratin
Collagen
Melanin
Lipid
Carbohydrate
Respiration
Biotransformation
Vitamin D

Source: Monteiro-Riviere, N.A. in *Dermal and Ocular Toxicology: Fundamentals and Methods*, CRC Press, Boca Raton, FL, 1991. With permission.

nonmembrane bound electron dense keratohyalin granules are present and contain a structural protein known as profilaggrin, a precursor of filaggrin. It has been speculated that these granules are involved in keratinization and formation of the barrier function of the skin.

The lamellar or membrane-coating granules (Odland bodies, lamellated bodies) containing stacks of lamellar disks are found within the stratum granulosum and increase in number and size as they approach the surface. As epidermal differentiation progresses, the lipid is synthesized and packaged into lamellar granules. These granules fuse with the cell membrane to release their lipid contents by exocytosis into the intercellular space between the stratum granulosum and stratum corneum layers (Yardley and Summerly, 1981; Matolsty, 1976). The granules then undergo a biochemical and physical change to form the lipid sheets that constitute the permeability barrier. Extraction of lipids in skin has shown that the epidermal lipid composition dramatically changes as the keratinocytes differentiate. Lipid composition of the epidermis may consist of phospholipids, glucosylceramides, ceramides, cholesterol, free fatty acids, triacylglycerols, and sphingosine (Downing, 1992; Swartzendruber et al., 1989).

In exceptionally thick skin and hairless regions of the body, such as the plantar and palmar surfaces, a stratum lucidum layer is present. This is a thin, translucent, homogeneous line between the stratum granulosum and stratum

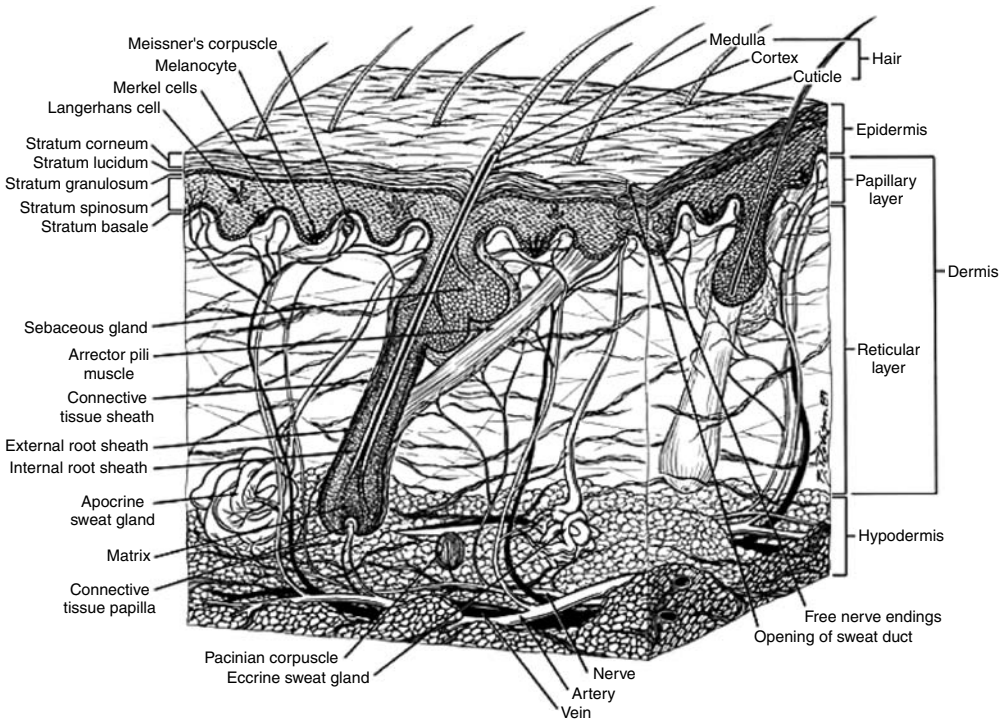


FIGURE 4.1 Schematic diagram illustrating the structure of mammalian skin (human and animal) from various regions of the body. (Reprinted from Monteiro-Riviere, N.A., in *Dermal and Ocular Toxicology: Fundamentals and Methods*, CRC Press, Boca Raton, FL, 1991. With permission.)

corneum, which consists of fully keratinized, closely compacted, dense cells that lack nuclei and cytoplasmic organelles. This translucent area contains a semifluid substance known as eleiden, which is similar to keratin but has a different staining pattern, as well as different protein-bound phospholipids (Leeson and Leeson, 1976). The outermost and final layer is the stratum corneum, which consists of several layers of completely keratinized dead cells, devoid of nuclei and cytoplasmic organelles that are constantly being shed. The most superficial layer of the stratum corneum is sometimes referred to as the stratum disjunctum. The thickness of the stratum corneum or number of cell layers varies depending on site and species (Monteiro-Riviere et al., 1990). It is this layer that provides an efficient barrier against transcutaneous water loss. Predominantly, it is the intercellular lipids, arranged into lamellar sheets that constitute the epidermal permeability barrier. Ruthenium tetroxide postfixation allows the visualization of these lipid lamellae at the ultrastructural layer (Swartzendruber, 1992). The number of lamellae may vary within the same tissue specimen. In some areas, it consists of a pattern of alternating electron-dense and electron-lucent bands that represent paired bilayers formed from fused lamellar granule disks as postulated by Landmann (Landmann, 1986; Swartzendruber et al., 1987, 1989; Madison et al., 1987).

It is widely acknowledged that the rate-limiting barrier to the absorption of most topically applied chemicals is the stratum corneum. Anatomical factors discussed such as the number of epidermal cell layers and the thickness of the stratum corneum may be parameters that modulate absorption. However, with knowledge that the pathway through the stratum corneum is via the intercellular lipids, the real resistance to absorption should relate to the length of this pathway. This has been clearly visualized using mercuric chloride staining in passive (Bodde et al., 1991) and iontophoretic (Monteiro-Riviere et al., 1994) drug delivery. Extraction of epidermal lipids using organic solvents reduces barrier function (Monteiro-Riviere et al., 2001; Hadgraft, 2001). The length is a function of the geometry of packing of the cells in the stratum corneum. The major route of a compound is via the intercellular bilipid channels, therefore, the absorption of a compound should be based on its diffusion path length (300–500 μm), not the actual thickness. Previous authors have described this spatial organization of vertical columns of interdigitating stratum corneum cells as resembling a tetrakaidecahedron. This 14-sided polygonal structure provides a minimum surface–volume ratio, which allows for space to be filled by packing without interstices (Menton, 1976a,b; Mackenzie, 1975). Therefore, the length is a function of the number of cell layers, overall thickness, the cell size, and the tortuosity of this pathway (Williams and Riviere, 1995).

As cells move outward from the basal layer, they undergo keratinization, which is the process by which epidermal cells differentiate. After the basal cells undergo mitosis, they migrate upward, increase in size, and produce large numbers of differentiation products (tonofilaments, keratohyalin granules, and lamellated bodies). Then the nuclei and organelles disintegrate, the filaments and keratohyalin arrange themselves into bundles, and the lamellated granules discharge their contents into the intercellular space coating the cells. The endpoint of this keratinization process is a nonviable, protein-rich terminally differentiated cell with a thicker plasmalemma, containing fibrous keratin and keratohyalin, surrounded by the extracellular lipid matrix. This forms the so-called "brick and mortar" arrangement, which is the morphological basis for the heterogeneous, two-compartment stratum corneum model (Elias, 1983).

The basement membrane is synthesized by the basal cells and separates the epithelium from the underlying connective tissue. This thin extracellular matrix is complex and consists of a highly organized structure of large macromolecules. By transmission electron microscopy, the cutaneous basement membrane is composed of four major components: (1) the cell membrane of the basal epithelial cell, (2) an electron lucent area beneath the plasma membrane called the lamina lucida, (3) an electron dense area beneath the lamina lucida called the lamina densa, and (4) the subbasal lamina containing anchoring fibrils, microfibril-like elements, and single-collagen fibers (Briggaman and Wheeler, 1975). In addition to this ultrastructural characterization, new epidermal–dermal junction biochemical components are constantly being identified and characterized. Representative examples of the most common ones include type IV collagen, laminin, entactin/nidogen, bullous pemphigoid antigen, heparan sulfate proteoglycan, fibronectin, GB3 (Nicein, BM-600, epiligrin), L3d (Type VII), 19-DEJ-1 (Uncein), epidermolysis bullosa acquisita, and the list is still growing. (Timpl et al., 1983; Woodley et al., 1984; Verrando et al., 1987; Fine et al., 1989; Rusenko et al., 1989; Briggaman, 1990). Many functions have been attributed to the basement membrane including a role in maintaining epidermal–dermal adhesion, and acting as a selective barrier between the epidermis and the dermis that restricts some molecules and permits the passage of others. In disease, they can also serve as a target for both immunologic and nonimmunologic injury.

4.3 DERMIS

The dermis lies beneath the basement membrane and consists primarily of dense irregular connective tissue within a matrix of collagen, elastic, and reticular fibers and is embedded in an amorphous ground substance made up of various types of proteoglycans. The predominant cell types of the dermis are fibroblasts, mast cells, and macrophages. In addition, plasma cells, fat cells, chromatophores, and extravasated leukocytes are often found. Blood vessels, lymphatics, and nerves traverse through the dermis along with glandular structures such as sebaceous glands, sweat glands, and hair follicles. There are two types of sweat glands in man: eccrine and apocrine. The eccrine gland is found over the entire body, except for a few areas where the apocrine gland may dominate (e.g., axilla, areola, pubis, perianal, eyelid, and external auditory meatus). In contrast, the apocrine gland is found over the entire body surface in hairy mammals and most carnivores.

The papillary layer, the most superficial layer of the dermis, conforms to the stratum basale layer and consists of loose connective tissue that blends into the deeper reticular layer, which consists of dense connective tissue. The hypodermis or subcutaneous layer is beneath the dermis and anchors the dermis to the underlying muscle or bone.

4.4 REGIONAL AND SPECIES DIFFERENCES

Studies in dermatology, cutaneous pharmacology, and toxicology involve experiments in which skin from different animal species and body regions are utilized. However, differences in stratum corneum thickness or number of cell layers must not be overlooked because they may affect barrier function. Table 4.2 summarizes the thickness of the nonviable stratum corneum and viable epidermis and the number of cell layers from the back (thoracolumbar area) of nine species used in dermatology research (Monteiro-Riviere et al., 1990). This finding confirms that skin thickness of the back was different across these species suggesting that body site alone is not a sufficient factor to ensure successful interspecies extrapolations. For more detail information regarding thickness of other body site measurements of these nine species, see Monteiro-Riviere et al. (1990). Some studies report differences in human skin but the database has only been collected at a few specific body sites (Blair, 1968). Measurements have been done on the number of cell layers at different sites and ages in humans and showed considerable variation in different areas of the body (Southwood, 1955; Rushmer et al., 1966). In general, data on the thickness of the stratum corneum are limited. Studies with human skin show that the stratum corneum in a given region is variable in both thickness and number of cell layers and that the sample length of the stratum corneum measured from a region is not consistent in thickness and number of cell layers (Holbrook and Odland, 1974).

TABLE 4.2
Comparative Thickness of the Epidermis and Number of Cell Layers from the Back of Nine Species

	Epidermis (μm)	Stratum Corneum (μm)	Number of Cell Layers (μm)
Cat	12.97 ± 0.93	5.84 ± 1.02	1.28 ± 0.13
Cow	36.76 ± 2.95	8.65 ± 1.17	2.22 ± 0.11
Dog	21.16 ± 2.55	5.56 ± 0.85	1.89 ± 0.16
Horse	33.59 ± 2.16	7.26 ± 1.04	2.50 ± 0.25
Monkey	26.87 ± 3.14	12.05 ± 2.30	2.67 ± 0.24
Mouse	13.32 ± 1.19	2.90 ± 0.12	1.75 ± 0.08
Pig	51.89 ± 1.49	12.28 ± 0.72	3.94 ± 0.13
Rabbit	10.85 ± 1.00	6.56 ± 0.37	1.22 ± 0.11
Rat	21.66 ± 2.23	5.00 ± 0.85	1.83 ± 0.17

Note: Paraffin sections stained with hematoxylin and eosin; $n = 6$, mean ± S.E.

Source: Monteiro-Riviere et al., *J. Invest. Dermatol.,* 95, 582–586, 1990. With permission.

Regional variations have been shown to affect percutaneous penetration in man (Feldman and Maibach, 1967; Maibach et al., 1971; Wester et al., 1980; Wester and Maibach, 1983). Studies with radiolabeled pesticides, parathion, malathion, and carbaryl were used to explore the permeability at 13 different anatomic sites in man. The palms and forearm showed a similar penetration rate but the abdomen and the dorsum of the hand had twice the penetration compared to the forearm (Maibach et al., 1971). Variations in percutaneous penetration of ^{14}C hydrocortisone demonstrate that the rate of absorption through back skin was more rapid than the flexor surfaces of the forearm (Feldman and Maibach, 1967). Additional studies using steady-state diffusion cells to investigate the absorption of phenol, methanol, octanol, caffeine, and aspirin through abdominal skin have shown that permeability of human abdominal skin within and between individuals was less variable than from other anatomic sites (Southwell et al., 1984). Similar studies have been conducted in other species. In the pig, absorption of topically applied parathion was greater for the back than the abdomen (Qiao et al., 1994). The extent and pattern of biotransformation were also different between these two sites (Qiao and Riviere, 1995). For parathion absorption, the ventral abdomen most closely resembles the human ventral forearm. The percutaneous absorption of methyl salicylate and the nerve agent VX was significantly greater when applied to the ear versus the epigastrium of pigs (Duncan et al., 2002). This finding questions the validity of using pig ears as an *in vitro* model to predict human absorption, and underscores the importance of anatomical differences in skin from different body regions on chemical absorption. Unfortunately, similar data are not available for many other compounds and species combinations.

Regional differences in total lipid content and lipid composition may occur within the stratum corneum at different anatomical sites. Sphingolipids and cholesterol are higher in the palmar and plantar stratum corneum than in the extensor surfaces of the extremities (Lampe et al., 1983). The distribution of lipids in nonkeratinized buccal epithelium is different than in keratinized areas due to the higher water permeability. Buccal epithelium contains glucosylceramides, acylceramides, small amounts of ceramides, but no acylglucosylceramides (Squier and Hall, 1985). In keratinized and nonkeratinized porcine oral epithelia, phospholipids are present in greater amounts than in the epidermis (Wertz, 1986). Also, there are differences in lipid composition in different species. Squalene is the major component in human skin, although most animal species have substantial amounts of diester waxes (Nicolaides et al., 1968). For an excellent review on the structure and function of mammalian epidermal lipids, see Wertz and Downing (1991).

Species differences in absorption for numerous chemicals have been well studied and adequately reviewed elsewhere (Wester and Maibach, 1975a,b, 1976, 1977). In general, the best animal models for human absorption are the domestic pig and the nonhuman primate. Evaluation of skin permeability was also performed on various animals by comparing the percutaneous penetration of nine radiolabeled compounds.

Statistically significant correlations to human data were obtained for the weanling pig and human skin–grafted nude mouse models. In contrast, significant correlations were not obtained in the hairless dog, pig skin–grafted nude mouse, and the nude mouse (Reifenrath et al., 1984).

Differences in the permeability of nicorandil, a coronary vasodilator, was determined in six different species (hairless mouse, hairless rat, guinea pig, dog, pig, and human), with the pig and human similar. It was suggested that the difference in permeability could be explained by the differences in species-specific skin surface lipids that may also affect the partitioning of nicorandil from the vehicle to the stratum corneum (Sato et al., 1991). These findings support the use of the pig as an optimal animal model for predicting human absorption.

Lipid extraction can have a profound effect by removing the essential lipids that are necessary for barrier function (Hadgraft, 2001; Monteiro-Riviere et al., 2001). The technique of altering the lipid composition has been applied to modulate skin permeability. Removal of lipids within the stratum corneum causes an increase in transepidermal water loss (TEWL) and also allows for an increase in the penetration of compounds. This enhancement of a compound is the basis for more efficient delivery of therapeutic compounds. Extraction of lipids from pig skin in three different body regions: the abdominal, inguinal, and back using three different solvent extraction procedures or tape stripping demonstrated that the mean total lipid concentration depended on the type of extraction solvents and body region. This was reproducible across sites and regions. Relative proportions of individual lipids (ceramides1-6, cholesterol, fatty acids, triglycerides, and cholesterol esters) extracted were similar across the three body regions, but higher concentrations of total lipids were extracted from the back (Monteiro-Riviere et al., 2001). Studies have also shown that there are regional differences in water content of human skin measured by Fourier transform near infrared (FT-NIR) spectroscopy. The results showed that these differences arise due to differences in depth, differences in specular reflection at the surface, and thickness of the stratum corneum (Egawa et al., 2006).

4.5 HAIR FOLLICLES

The basic architecture of the integument is similar in all mammals. However, structural differences in the arrangement of hair follicles and hair follicle density exist between domestic and laboratory animals. The hair density in pig and human is sparse compared to that of the rodent. The skin from the back of pigs and the abdomen of humans have 11 ± 1 hair follicles/cm^2, in comparison to the back of the rat with 289 ± 21, the mouse with 658 ± 38, and the hairless mouse with 75 ± 6 (Bronaugh et al., 1982). For a comprehensive review of hair follicle arrangement and microscopic anatomy of the integument in different domestic species, see Monteiro-Riviere (2006) and Monteiro-Riviere (1991).

Hair follicles, sebaceous glands, and sweat glands are often envisioned as special channels through the stratum corneum that facilitates absorption of topical compounds, thus bypassing the rate-limiting stratum corneum barrier. Controversy exists over the significance of these hair follicle pathways in percutaneous absorption. The comparative permeability of human and animal skin may be related to diffusion of compounds through appendages in the skin. Numerous studies have been designed to test this hypothesis. One must always remember that even when a compound traverses the skin via hair follicles, passage through the stratum corneum still occurs. It is probable that any increase in absorption attributed to the appendages probably results from the increased surface area seen in the attending invaginations of the stratum corneum, therefore areas covered with hair have a greater skin surface area available for transdermal absorption. Hair follicle delivery has been considered a way to develop treatments for hair follicle–associated diseases, gene therapy, and immunotherapy.

Hair follicle–rich areas such as the scalp, angle of jaw, postauricular area, and forehead have been shown to allow greater penetration of some pesticides (Maibach et al., 1971). The importance of hair follicles in percutaneous absorption was evaluated in a model of regrown skin without hair follicles dorsally on the hairless rat. Diffusion cell studies were used to compare the absorption of tritiated hydrocortisone, niflumic acid, caffeine, and *p*-aminobenzoic acid in intact- and appendage-free skin. These studies confirmed a higher rate of diffusion in intact skin and suggested that hair follicles acted as the major absorption pathway (Illel et al., 1991). Other investigators have also studied this phenomenon. Flow through organ culture studies were performed on normal-haired and hairless mice with benzo[a]pyrene and testosterone. The overall permeation of testosterone was greater than that of benzo[a]pyrene, and showed no strain differences. However, benzo[a]pyrene absorption was higher in the haired mice than the hairless mice.

Additional studies of three phenotypic hair-density variants, suggest that the permeability of both compounds was the highest in the haired phenotype, lowest in the hairless phenotype, and intermediate in the fuzzy-haired animal. They concluded that transappendageal penetration contributes significantly to overall skin absorption (Kao et al., 1988). Hence, regional distribution of skin appendages could influence absorption of some compounds. Absorption of estradiol and progesterone was studied on normal and appendage-free (scar) hairless rats to determine if differences were due to lack of appendageal structures or modification of blood flow. Concentration of both steroids was significantly higher in normal than in scar tissue (Hueber et al., 1994a). They also compared the absorption of estradiol, hydrocortisone, progesterone, and testosterone through scar skin (without hair follicles, sebaceous glands, and sweat glands) from the abdomen or mammary areas of humans. Again, absorption was significantly higher in normal than scar tissue. Based on these findings, hair follicles and sebaceous glands may constitute a route for penetration for these steroids (Hueber et al., 1994b). Another study tested the appendageal density and absorption of the vasodilator methyl nicotinate, on the forehead, forearm, and palms of

humans. Penetration was the greatest on the forehead, intermediate on the forearm, and least through the palms. They also concluded that there was a correlation between methyl nicotinate absorption and appendageal density (Tur et al., 1991). Other investigators have found that the penetration process of a compound is dependent on the phase of the hair growth cycle, sebum production, and the state of the follicular infundibulum (Lademann et al., 2001). However, there is another interpretation to these data. In areas with dense hair, limited interfollicular regions (areas without hair) of skin are present. Therefore, the compound appears to penetrate only through hair follicles. This factor, coupled with the increased stratum corneum surface area associated with the invaginations of follicles, may explain the enhanced penetration seen in hairy skin.

Studies have also shown that there are racial disparities in absorption in humans due to the follicular density and follicular reservoir that may play a role in the penetration of drugs and cosmetics. The hair follicle density of Asians is less than African Americans for the forehead region. Lower values were detected for volume, surface, follicular orifice, and hair shaft diameter on the thigh and calf regions for Asians and African Americans. The follicular volume was greater for Caucasians (Mangelsdorf et al., 2006). These types of differences must be considered when designing skin absorption studies among different ethnic populations. In summary, hair follicle density, stratum corneum thickness, number of cell layers, and lipid composition are important structural variables to be considered when comparing absorption across different body sites or species.

4.6 BLOOD FLOW

The complexity of blood vessels in the skin is limited to the dermis, for the epidermis is avascular. Large arteries arise from a network in the subcutaneous layer, which send some of their branches to the superficial and deep dermis. The superficial arteries traverse through the dermis and send smaller branches that supply hair follicles, sebaceous glands, and sweat glands. A network of smaller arteries, the rete subpapillare (horizontal plexus), run between the papillary and reticular layers. Small arterioles from this plexus supply the capillary loops (subepidermal plexus) in the dermal papillary layer. Beneath the basement membrane, capillary beds are present in the matrix of hair follicles and around sebaceous and sweat glands. In specific body sites (fingertips, toes, lips, and nose), alternative channels called arteriovenous anastomoses or shunts are present, which allow blood to be passed from the arteriole to the venule. When connective tissue surrounds such a vascular structure, it is referred to as a glomus, which functions in regulation of body temperature and peripheral blood circulation. For a complete understanding about the cutaneous vasculature, see Ryan (1991).

Most research models in dermatology, cutaneous pharmacology, and reconstructive surgery have been animals. It is important to be aware of the anatomic and physiologic differences in blood flow between species and sites within species before the variable of blood flow can be used to explain percutaneous absorption. In humans, laser Doppler velocimetry (LDV) is often used to assess cutaneous blood flow. LDV is an accepted noninvasive technique that assesses relative cutaneous capillary blood perfusion (Holloway and Watkins, 1977; Young et al., 1985; Fischer et al., 1983). It has been used to assess the vascular response in man to acute inflammation (Holloway, 1980; Ross et al., 1987; Serup and Staberg, 1985), heat (Holloway, 1980), ultraviolet light (Young et al., 1985; Frodin et al., 1988), corticosteroids (Bisgaard et al., 1986), nitroglycerin (Sunberg, 1984), minoxidil (Wester et al., 1984), to determine depths of superficial, deep dermal, and subdermal burns (Micheels et al., 1984; Alsbjorn et al., 1984), as well as to evaluate donor sites in reconstructive surgery (Goldberg et al., 1990).

Skin blood flow measurements using LDV at various sites in humans showed interindividual and spatial variations (Tur et al., 1983). The magnitude of cutaneous blood flow and epidermal thickness has been postulated to explain the regional differences in percutaneous absorption between body sites in man and animals. A comprehensive study comparing the histologic thickness (Table 4.2) and laser Doppler blood flow measurements (Table 4.3) was performed at five cutaneous sites (buttocks, ear, humeroscapular joint, thoracolumbar junction, and abdomen) in nine species (cat, cow, dog, horse, monkey, mouse, pig, rabbit, and rat) to determine the correlation of blood flow and thickness. These studies strongly suggested that LDV blood flow and skin thickness did not correlate across species and body sites but are independent variables that must be evaluated separately in dermatology, pharmacology, and toxicology studies (Monteiro-Riviere et al., 1990).

The role of the cutaneous vasculature was studied in the topical delivery of ^{3}H piroxicam, a nonsteroidal antiinflammatory drug. Dermal penetration of ^{3}H piroxicam gel was evaluated by *in vitro* diffusion cells and *in vivo* (pigs) at two different tissue beds: one that is vascularized by direct cutaneous and the other by musculocutaneous arteries. The *in vitro* fluxes were identical indicating a similar rate of stratum corneum and epidermal absorption, however more extensive and deeper tissue penetration was noted at the musculocutaeous sites. This suggests that the vascular anatomy is important in determining the extent of dermal penetration (Monteiro-Riviere et al., 1993). Other studies also implied that topical administration in male rats resulted in a high concentration of the drug in the underlying vasculature that could not be attributed to redistribution via the systemic circulation (McNeill et al., 1992). Thus, the cutaneous vasculature does not function as an infinite sink that removes all topically applied drugs to the systemic circulation (Riviere and Williams, 1992). This mechanism of drug delivery has been alluded by other investigators (Wada et al., 1982; Torrent et al., 1988; Guy and Maibach, 1983). Also, we have demonstrated that local modulation of the vasculature by coiontophoresis of vasoactive compounds could affect drug distribution to the underlying tissue (Riviere et al., 1991). All of these studies suggest a major role for the cutaneous

TABLE 4.3
Blood Flow Measurements of Comparative Species at Five Cutaneous Sites (Mean ± SE)

Species	BUT	EAR	HSJ	TLJ	VAB
Cat	1.82 ± 0.59	6.46 ± 2.30	1.86 ± 0.70	2.39 ± 0.35	6.19 ± 0.94
Cow	6.03 ± 1.84	6.98 ± 2.19	5.51 ± 2.32	5.49 ± 1.49	10.49 ± 2.13
Dog	2.21 ± 0.67	5.21 ± 1.53	5.52 ± 1.31	1.94 ± 0.27	8.78 ± 1.40
Horse	3.16 ± 1.22	–	6.76 ± 1.49	2.99 ± 0.86	8.90 ± 1.46
Monkey	3.12 ± 0.58	20.93 ± 5.37	8.49 ± 3.28	2.40 ± 0.82	3.58 ± 0.41
Mouse	3.88 ± 0.92	1.41 ± 0.48	10.10 ± 3.51	20.56 ± 4.69	36.85 ± 8.14
Pig	3.08 ± 0.48	11.70 ± 3.02	6.75 ± 2.09	2.97 ± 0.56	10.68 ± 2.14
Rabbit	3.55 ± 0.93	8.38 ± 1.53	5.38 ± 1.06	5.46 ± 0.94	17.34 ± 6.31
Rat	4.20 ± 1.05	9.13 ± 4.97	6.22 ± 1.47	9.56 ± 2.17	11.35 ± 5.53

Note: But = buttocks; Ear = pinnae; HSJ = humeroscapular joint; TLS = thoracolumbar junction; VAB = ventral abdomen.
Source: Monteiro-Riviere et al., *J. Invest. Dermatol.*, 95, 582–586, 1990. With permission.

vasculature in modulating absorption and dermal penetration of some topically applied drugs.

4.7 AGING

The anatomical and physiological changes in skin associated with aging may also affect the barrier function. Some of the major problems that complicate the understanding of aged skin is the ability to differentiate actinically damaged from chronically aged or age-related changes due to environmental influences (e.g., chronic sun exposure, cold, wind, low humidity, chemical exposure, or physical trauma), maturation process (e.g., newborn to adult), diseases, or hormonal changes associated with menopause.

Aging skin is usually generalized by a wrinkled and dry appearance. However, the microscopic changes in the epidermis associated with aging include flattening of the epidermal–dermal junction, retraction of epidermal downgrowths, thinness, reduction in number and output of sweat glands, and a more heterogeneous basal cell population (Rapaport, 1973; Lavker, 1979; Hull and Warfel, 1983; Gilchrest, 1984; Kligman et al., 1985; Lavker et al., 1986, 1987; Kligman and Balin, 1989). Numerous ultrastructural changes have also been documented in the dermis of aged skin. These include changes in the architecture of the elastic fiber framework (Lavker, 1979; Montagna and Carlisle, 1979), dermal shrinkage (Evans et al., 1943), elastic fiber disintegration, thickening and clumping (Braverman and Fonferko, 1982a), progressive rise in the modulus of elasticity (Grahame and Holt, 1969), decrease in collagen content (Branchet et al., 1991), modification of collagen from fascicular to granular (Pieraggi et al., 1984), and a decrease in tensile strength (Vogel, 1983). Also, thickening of the vascular wall vessels (Braverman and Fonferko, 1982b), loss of melanocytes in the hair bulb, and fewer glands were observed (Gilchrest, 1984).

The general morphological organization and thickness of the stratum corneum in humans do not change with age (Lavker, 1979; Christophers and Kligman, 1965), but the lipid content and intercellular cohesion in the stratum corneum decrease with age (Leveque et al., 1984). Thin layer chromatography and photodensitometry were used to study interindividual differences in fatty acid composition of skin surface lipids. Both age and sex were significant factors (Nazzaro-Porro et al., 1979). Stratum corneum hydration parameters remain unchanged or slightly decreased with age (Potts et al., 1984). Premenopausal women tend to have smaller corneocytes than postmenopausal woman (Fluhr et al., 2001). All of these alterations may alter the clearance and absorption of transdermally absorbed compounds through and from the skin. This can best be illustrated with a few examples.

A dose response study of 14 different pesticides on young and adult female rats showed significant age-dependent differences in skin penetration in 11 pesticides (Hall et al., 1988). A decrease in dermal absorption of some chemicals in the aged may be due to morphological differences in blood flow (Christophers and Kligman, 1965). Studies in male rats, 1–24 months, and mice, 1–22 months of age, showed that blood flow in mice increased between 1 and 2 months, remained constant to 19 months, then increased at 22 months; but the blood flow in rats was constant except at 2 months. Also, the number of viable epidermal layers in mice was constant, while in rats it decreased with age. Epidermal thickness in both mice and rats decreased from 2 to 3 months. Dermal thickness decreased from 3 to 22 months in mice, and increased in rats from 1 to 2 months (Monteiro-Riviere et al., 1991). Other studies have shown a decrease in the dermal absorption of Evans blue dye in old rats as compared to middle-aged rats (Kohn, 1969). Cardiac output declines with age and the pattern of blood flow distribution also changes with the proportion of cardiac output received by the kidneys, skin, gastrointestinal tract, and liver decreasing in older rats (Yates and Hiley, 1979). Therefore, reductions in blood flow could alter the distribution of compounds to and from these tissues. Age difference in blood flow can occur and should be considered when evaluating cutaneous toxicity studies in different aged animals.

A decrease was observed in TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin) and 4 PeCDF (2,3,4,7,8-pentachlorodibenzofuran) absorption in older rats compared to 10 weeks old rats (Banks et al., 1990). TCDD absorption decreased from 3 to 5 weeks in rats (Jackson et al., 1990). The decreased absorption of these compounds in older animals could be explained by decreased clearance from the application site due to reduced perfusion seen in rats older than 2 months. Maturational changes in the dermal absorption of TCDD in rats also indicated that TCDD is absorbed to a greater degree in young animals and a significant decrease in potential for systemic exposure occurs during maturation and aging (Anderson et al., 1993). Age-related differences in the absorption of 14 different pesticides studied in 1 and 3 months old rats showed an increase in some compounds and a decrease in others (Shah et al., 1987). Therefore, the physiological and physiochemical properties (e.g., lipophilicity, molecular size) of the compound may be as important in assessing percutaneous absorption in aged animals as anatomical or physiological differences. Other investigators studied the pharmacodynamic measurements of methyl nicotinate in aged individuals and showed no differences between young (20–34 years) and old (64–86 years) populations (Roskos et al., 1990). Studies with testosterone, estradiol, hydrocortisone, and benzoic acid in young controls (18–35 years), young-old (65–75 years), and old-old (>75 years) humans showed different patterns depending upon the compound. For estradiol, absorption in the old-old group was less than in the other two populations. For hydrocortisone and benzoic acid, absorption in the young was greater than both elderly groups. In contrast, testosterone was lowest in the young-old group (Roskos et al., 1986). These studies showed a tendency for decreased absorption with most compounds with advancing age.

Another study evaluating *tri-N*-propyl phosphate (TNPP) in *in vitro* human skin from various anatomic sites ranging from 3 to 57 years showed a decrease in permeability with increased age (Marzulli, 1962). Studies involving the percutaneous absorption of 2-*sec*-butyl-4,6-dinitrophenol (dinoseb) in relation to age and dosage in *in vitro* and *in vivo* rat skin showed that dermal absorption in young rats was less than in adults at all doses studied (Hall et al., 1992). These studies have many additional variables making simple extrapolations difficult. For example, hair follicle growth cycle and body site were significant factors in hydrocortisone absorption studies in rats (Behl et al., 1984). Many studies do not control for these factors. Therefore, in most studies conducted to date, age is an important multifaceted factor involving many biological processes (e.g., altered lipid composition, blood flow), which produce compound-dependent effects. These factors must be taken into account when interpreting any study.

4.8 DISEASES

Skin permeability may be increased or decreased depending on the condition of the skin. Impaired skin barrier function may be the cause of some dermatitis conditions. Common diseases such as psoriasis may influence absorption. Psoriasis may be defined as having an accelerated rate of epidermal cell replication, a decrease in tonofilaments and keratohyalin granules, a disordered, loosely and irregularly stacked stratum corneum, and tortuous and dilated capillary loops (Braverman et al., 1972). These abnormal capillaries (e.g., large lumen, fenestrations between endothelial cells, and multilayered basement membrane) may have an affect on blood flow to skin. In addition, intercellular ionic calcium distribution is different in psoriatic skin that could alter lipid permeability (Menon and Elias, 1991). For example, hydrocortisone has been shown to increase absorption in psoriatic skin (Schaefer et al., 1977; Zesch et al., 1975).

In addition, other skin diseases such as essential fatty acid deficiency and ichthyosis may have an effect on compound penetration. Epidermal barrier function is altered by abnormal lipid composition in noneczematous atopic dry skin (Fartasch et al., 1992). Any disease process that has altered any of the above mentioned anatomical factors would be expected to modulate chemical percutaneous absorption. The challenge to researchers is to define the specific factor that has the greatest impact on a specific chemical's toxicology or clinical efficacy.

4.9 CONCLUSIONS

This chapter should provide the reader with a brief introduction to the effects of anatomy on percutaneous absorption that is summarized in Figure 4.2. There are numerous factors, which must be taken into consideration when designing experiments or interpreting data. Many different processes such as aging, disease, or chemical damage may affect specific anatomical components of skin. Anatomy is a useful framework upon which to classify the various biological processes that may be affected. The critical anatomical variables that are important for influencing the absorption of compounds across the skin include those factors governing the length of the intercellular pathway (thickness, cell

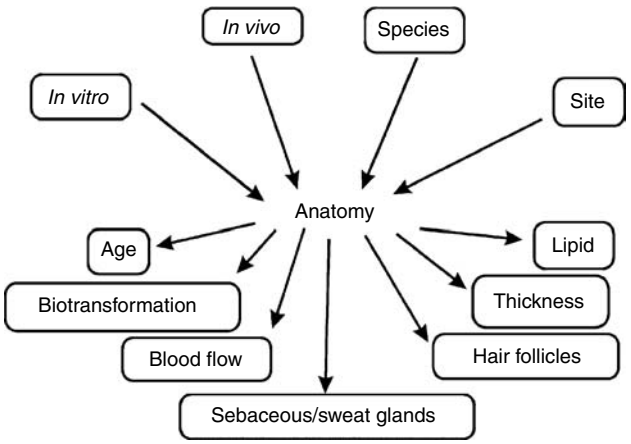


FIGURE 4.2 Overall summary depicting the anatomical factors that can influence absorption.

layers, cell size, and tortuosity), lipid composition of this medium, potential appendageal shortcuts (hair follicle density, sebaceous and sweat glands), and the nature and density of the underlying dermal circulation. A knowledge of how a process such as aging or disease affects each structure's integrity, then gives one a perspective on how the absorption of a chemical may be modified. Changes in these factors secondary to species, body regions, age, or disease would be expected to affect overall absorption. A major complication in most cases is that more than one component is altered and the effect observed is very dependent on the specific chemical's physiochemistry.

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5 Percutaneous Penetration Enhancers: Overview

Haw-Yueh Thong, Hongbo Zhai, and Howard I. Maibach

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5.1 INTRODUCTION

Skin is an optimal interface for systemic drug administration. Transdermal drug delivery (TDD) is the controlled release of drugs through intact or altered skin to obtain therapeutic levels systematically and to affect specified targets for the purpose of, for example, blood pressure control, pain management, and others. Dermal drug delivery (DDD) is similar to TDD except that the specified target is the skin itself (Kydonieus and Wille, 2000). TDD has the advantages of bypassing gastrointestinal incompatibility and hepatic "first pass" effect; reduction of side effects due to the optimization of the blood concentration–time profile; predictable and extended duration of activity; patient-activated/patient-modulated delivery; elimination of multiple-dosing schedules thus enhancing patient compliance; minimization of inter- and intrapatient variability; reversibility of drug delivery allowing the removal of drug source; and relatively large area of application comparing with the mucosal surfaces (Kydonieus and Wille, 2000).

After nearly four decades of extensive study, the success of this technology remains limited, with many problems waiting to be solved, one of which is the challenge of low skin permeability hindering the development of TDD for macromolecules. To overcome the skin barrier safely and reversibly, while enabling the penetration of macromolecules, is a fundamental problem in the field of TDD and DDD.

Several technological advances have been made in the recent decades to overcome skin barrier properties (Smith and Maibach, 2005). Examples include physical means such as iontophoresis, sonophoresis, microneedles; chemical means using penetration enhancers (PE); and biochemical means such as liposomal vesicles and enzyme inhibition.

We overview physical and biochemical means of penetration enhancement and focus on the common chemical PEs. We discuss the classification and mechanisms of chemical PEs, its applications in TDD, and trends and development in penetration enhancement.

5.2 PHYSICAL PENETRATION ENHANCEMENT

Physical means of penetration enhancement mainly incorporate mechanisms to transiently circumvent the normal barrier function of stratum corneum (SC) and to allow the passage of macromolecules. Although the mechanisms are different, these methods share the common goal to disrupt SC structure to create "holes" big enough for molecules to permeate. Table 5.1 summarizes the commonly investigated technologies of physical penetration enhancement. Two of the better-known technologies are iontophoresis and sonophoresis, and the "holes" created by these methods are generally believed to be of nanometer dimensions permissive of transport of small drugs (Prausnitz, 2004). A new and exciting technology for macromolecule delivery is microneedle-enhanced delivery. These systems use arrays of tiny needlelike structures to create transport pathways of microns dimensions, and should be able to permit transport of macromolecules, possibly supramolecular complexes and microparticles. These systems have greatly enhanced (up to 100,000-fold) the penetration of macromolecules through

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TABLE 5.1
Physical Methods of Penetration Enhancement

Method	Definition	Mechanism(s)	Examples of Drugs	References
Iontophoresis	The electrical driving of charged molecules into tissue by passing a small direct current through a drug-containing electrode in contact with skin	1. Electrical repulsion from the driving electrode drives charged molecules 2. The flow of electric current enhances skin permeability 3. Electroosmosis affects uncharged and large polar molecules	Calcitonin, transnail delivery of salicylic acid, transdermal delivery of peptides, proteins, and oligonucleotides	Santi et al. (1997), Narasimha (2007), Barry (2001), Miller et al. (1990), Mitragotri et al. (1995)
Electroporation	A method of reversibly permeabilizing lipid bilayers by the application of an electric pulse	Application of short (micro- to millisecond) electrical pulses of ~100–1000 V/cm create transient aqueous pores in the lipid bilayers	Methotrexate, timolol, fentanyl, tetracaine, nalbuphine, cyclosporin-A	Wong et al. (2005), Denet and Preat (2003), Hu et al. (2000), Sung et al. (2003), Vanbever et al. (1996a,b), Wang et al. (1998)
Sonoporation	Ultrasound mediated delivery of therapeutic agents into biological cells	1. Low energy frequency disturbs the lipid packing in SC by cavitation 2. Shock waves increase free volume space in bimolecular leaflets, thus enhance permeation	Insulin, cutaneous vaccination, transdermal heparin delivery, transdermal glucose monitoring, delivery of acetyl cholinesterase inhibitors for the treatment of Alzheimer's disease, treatment of bone diseases and Peyronie's disease, and dermal exposure assessment	Boucaud et al. (2002), Barry (2001), Vranic (2004)
Microneedle-enhanced delivery systems	A method using arrays of microscopic needles to open pores in SC, thus facilitating drug permeation	Bypass the SC and deliver drugs directly to the skin capillaries. Also has the advantage of being too short to stimulate the pain fibers	Oligonucleotide, insulin, protein vaccine, DNA vaccine, methyl nicotinate	Prausnitz (2004), Sivamani et al. (2005)

Source: Zhai, H. and Maibach, H.I., *Skin Pharmacol. Appl. Skin Physiol*., 14 (1), 2001. With permission.

skin (Barry, 2001), although offering painless drug delivery (Kaushik et al., 2001; Sivamani et al., 2005).

5.3 BIOCHEMICAL PENETRATION ENHANCEMENT

Biochemical means of penetration enhancement include using prodrug molecules (Sloan and Bodor, 1982), chemical modification (Choi et al., 1990), enzyme inhibition (Morimoto et al., 1992), and the usage of vesicular systems or colloidal particles (Mezei and Gulasekharam, 1980). Among these strategies, special formulation approaches based mainly on the usage of colloidal carriers are most promising. Liposomes (phospholipids-based artificial vesicles) and niosomes (nonionic surfactant vesicles) are widely used to enhance drug delivery across the skin. In addition, proliposomes and proniosomes, which are converted into liposomes and niosomes upon simple hydration are also used in TDD (Choi and Maibach, 2005). Generally, these colloidal carriers are not expected to penetrate into viable skin. Most reports cite a localizing effect whereby the carriers accumulate in SC or other upper skin layers (Barry, 2001).

More recently a new type of liposomes called transferosomes has been introduced (Planas et al., 1992; Cevc, 1996). Transferosomes consist of phospholipids, cholesterol and additional "edge activators"—surfactant molecules such as sodium cholate. The inventors claim that 200–300 nm-sized transferosomes are ultradeformable and squeeze through pores less than one-tenth of their diameter, and are thus able to penetrate intact skin. Penetration of these colloidal particles works best under *in vivo* conditions and requires a hydration gradient from the skin surface toward the viable tissues to encourage skin penetration under nonoccluded conditions.

In addition, ethosomes, which are liposomes high in ethanol content (up to 45%), penetrate skin and enhance compound delivery to deep skin strata or systematically. The mechanism suggested is that ethanol fluidizes both ethosomal lipids and lipid bilayers in the SC, allowing the soft, malleable vesicles to penetrate through the disorganized lipid bilayers (Touiton et al., 2000).

In general, six potential mechanisms of actions of these colloidal carriers were proposed (Barry, 2001):

1. Penetration of SC by a free drug process—drug releases from vesicle and then penetrates skin independently.
2. Penetration of SC by intact liposomes.

3. Enhancement due to release of lipids from carriers and interaction with SC lipids.
4. Improved drug uptake by skin.
5. Different enhancement efficiencies control drug input.
6. The role of protein requires elaboration.

5.4 CHEMICAL PENETRATION ENHANCERS (CPEs)

Substances that help promote drug diffusion through the SC and epidermis are referred to as penetration enhancers (PEs), accelerants, adjuvants, or sorption promoters (Pfister et al., 1990). PEs have been extensively studied given its advantages such as design flexibility with formulation chemistry, patch application over large area. PEs improve drug transport by reducing the resistance of SC to drug permeation. To date, none of the existing CPEs has proven to be ideal. In particular, the efficacy of PEs toward the delivery of high molecular weight drugs remains limited. Attempts to improve enhancement by increasing the potency of enhancers inevitably lead to a compromise on safety issues. Achieving sufficient potency without irritancy has proved challenging.

5.4.1 Classification of CPEs

The diverse physicochemical properties and variation in mechanisms of action of compounds investigated for their penetration enhancement effects made a simple classification scheme for PEs difficult to set up. Hori et al. (1990) proposed a conceptual diagrammatic approach based on Fujita's (1954) data for the classification of PEs. In this approach, they determined organic and inorganic values for PEs, and the resultant plot of organic versus inorganic characteristics grouped PEs into distinct areas on the diagram—area I encloses enhancers, which are solvents, area II designates PEs for hydrophilic drugs, and area III contains PEs for lipophilic compounds. However, Lambert et al. (1993) grouped most PEs into three classes: solvents and hydrogen bond acceptors (e.g., dimethylsulfoxide, dimethylacetamide, and dimethylformamide), simple fatty acids and alcohols, and weak surfactants containing a moderately sized polar group (e.g., Azone®, 1-dodecylazacycloheptan-2-one); whereas Pfister et al. (1990) classified PEs as either polar or nonpolar. To date, there is no consensus as to which classification to adopt. Table 5.2 classifies commonly investigated PEs based on the chemical classes to which the compounds belong (Barry, 1995). Only representative compounds are listed to avoid an exhaustive list. Note that a perfect classification is yet to be developed and the key lies in a comprehensive understanding of the mechanisms and the physicochemical parameters of CPEs.

5.4.2 Mechanism of CPEs

The mechanisms of action proposed for commonly seen CPEs are listed in Table 5.2. Basically, transdermal penetration of most drugs is a passive diffusion process (Hsieh, 1994). There are three major potential routes for penetration—appendageal (through sweat ducts or hair follicles with associated sebaceous glands), transcellular permeation through the SC, or intercellular permeation through the SC (Barry, 2001). The intact SC comprises the predominant route through which most molecules penetrate. On the other hand, despite its small available fractional area of 0.1%, the appendageal route, especially the follicular route, has recently received considerable attention and was found to be an important penetration pathway and a possible space for an intracutaneous reservoir (Schaefer and Lademann, 2001; Lademann et al., 2003; Otberg et al., 2004). Liposomal formulations have shown to be useful delivery systems for follicular drug targeting (Hoffmann, 1998), and transfollicular drug delivery seems promising for gene therapy and vaccination (Christoph et al., 2000; Hoffmann, 2000; Corsarelis, 2000; Hoffmann, 2005).

Kanikkannan et al. (2000) suggested three pathways for drug penetration through the skin: polar, nonpolar, and both. The mechanism of penetration through the polar pathway is to cause protein conformational change or solvent swelling; whereas the key to penetrate via the nonpolar pathway is to alter the rigidity of the lipid structure and fluidize the crystalline pathway. Some enhancers may act on both polar and nonpolar pathways by dissolving the skin lipids or denaturing skin proteins. However, Ogiso and Tanino (2000) proposed the following mechanisms for the enhancement effect: (a) an increase in the fluidity of the SC lipids and reduction in the diffusional resistance to permeants, (b) the removal of intercellular lipids and dilation between adherent cornified cells, (c) an increase in the thermodynamic activity of drugs in vehicles, (d) the exfoliation of SC cell membranes, the dissociation of adherent cornified cells and elimination of the barrier function.

Ogiso et al. (1995) also proposed examples of PEs with different relative enhancement capabilities due to differences in the chemical structure and other parameters. In their study, the relative ability to enhance transdermal penetration of indomethacin into hairless rat skin was studied. The results were summarized as follows (Chan, 2005):

Mechanisms	Comparison
Extraction of intercellalur lipids and dilations between cornified cells, permitting percutaneous passage of polar substances	1-dodecylazacycloheptane-2-one(Azone)>*n*-octanol>*d*-limonen>oleic acid>cineol
Increase in partitioning into skin	1-dodecylazacycloheptane-2-one>*n*-octanol>cineol>*d*-limonen>oleic acid>isopropyl myristate>monooleate
Increase in the fluidity of SC lipids and reduction in diffusional resistance	1-dodecylazacycloheptane-2-one>isopropyl mysirate>monoolein>oleic acid>cineol, sodium oleate
Increase in thermodynamic activity in vehicles	*n*-octanol>sodiumoleate>*d*-limonen>monoolein>cineol>oleyl oleate>isopropyl myristate

TABLE 5.2
Chemical Penetration Enhancers

Category and Examples	Cosolvent/Vehicle	Mechanism	Examples of Drugs (Ghosh et al., 1997)	Comment	References
Sulfoxides					
Dimethylsulfoxide (DMSO)		1. Increases lipid fluidity 2. Promotes drug partitioning	DMSO: theophylline, salicylic acid, hydrocortisone, testosterone, scopolamine, antimycotics, fluocinolone acetonide, flufenamic acid		Scheuplein and Blank (1971), Sekura and Scala (1988)
Decylmethylsulfoxide (DCMS)		Protein–DCMS interactions, resulting in a change in protein conformation, creating aqueous channels	DCMS: methotrexate, naloxone, pyridostigmine bromide, hydrocortisone, progesterone	DCMS enhances polar drug more effectively	
Alkanones					
N-heptane, *N*-octane, *N*-nonane, *N*-decane, *N*-undecane, *N*-dodecane, *N*-tridecane, *N*-tetradecane, *N*-hexadecane		Extensive barrier alteration of SC	Propanol, diazepam		Hori et al. (1991)
Alcohols					
Alkanol: ethanol (E), propanol, butanol, 2-butanol, pentanol, 2-pentanol, hexanol, octanol, nonanol, decanol, benzyl alcohol (BA) Fatty alcohol: caprylic, decyl, lauryl (LA), 2-lauryl, myristyl, cetyl, stearyl, oleyl, linoleyl, linolenyl alcohol		1. Low molecular weight alkanols (C≤6) may act as solubilizing agents 2. More hydrophobic alkanols may extract lipids from SC,* leading to increased diffusion	E: tacrine, metrifonate, dichlorvos, ketolorac, nitroglycerin, tazifylline, betahistine, cyclosporin A LA: buprenorphine		Tsuzuki et al. (1988), Friend et al. (1988), Ding et al. (2006), Liu et al. (2006), Aungst et al. (1986)
Polyols					
Propylene glycol (PG), polyethylene glycol (PEG), ethylene glycol, diethylene glycol, triethylene glycol, dipropylene glycol, glycerol (G), propanediol, butanediol , pentanediol, hexanetriol	43× enhancement of diazepam and 86× enhancement of midazolam maleate seen in PG and 5% Azone in a PG:E:water (2:2:1) vehicle	PG may solvate α-keratin and occupy hydrogen bonding sites, reducing drug-tissue binding	PG: 5-fluorouracil, tacrine, ketorolac, isosorbide dinitrate, clonazapem, albuterol, verapamil, betahistine, estradiol, dihydroergotamine, methotrexate, steroids, midazolam maleate, diazepam PEG: terbutaline G: diazepam, terbutaline, 5-fluorouracil	Inclusion of 2% Azone or 5% oleic acid to PG produced a more bioactive formulation	Mollgaard and Hoelgaard (1983), Herai et al. (2007)
Amides					
Urea, dimethylacetamide (DMA), diethyltoluamide, dimethylformamide (DMF), dimethyloctamide, dimethyldecamide		Urea: hydration of SC, keratolytic, creating hydrophilic diffusion channels DMA/DMF: (low concentration): partition to keratin, (high concentration): increase lipid fluidity, disrupt lipid packaging	Urea: ketoprofen, 5-fluorouracil DMA/DMF: griseofluvin, betamethasone 17-benzoate, caffeine	Urea analogues in PG enhanced permeability of 5-fluorouracil 6×	Aungst et al. (1986), Feldman and Maibach (1974)

Biodegradable cyclic urea: 1-alkyl-4-imidazolin-2-one			Indomethacin	Comparable to or better than Azone	Wong et al. (1988)
Pyrrolidone derivatives: 1-methyl-2-pyrrolidone (1M2P), 2-pyrrolidone, 1-lauryl-2-pyrrolidone, 1-methyl-4-carboxy-2-pyrrolidone, 1-hexyl-4-carboxy-2-pyrrolidone, 1-lauryl-4-carboxy-2-pyrrolidone, 1-methyl-4-methoxycarbonyl-2-pyrrolidone, 1-hexyl-4-methoxycarbonyl-2-pyrrolidone, 1-lauryl-4-methoxycarbonyl-2-pyrrolidone, *N*-methyl-pyrrolidone (NMP), *N*-cyclohexylpyrrolidone, *N*-dimethylaminopropylpyrrolidone, *N*-cocoalkypyrrolidone, *N*-tallowalkylpyrrolidone		Interact with both keratin in the SC and with lipids in the skin structure	1M2P: griseofulvin, theophylline, tetracycline, ibuprofen, betamethasone 17-benzoate NMP: prazosin		Aungst et al. (1986), Sasaki et al. (1991)
Biodegradable pyrrolidone derivatives: Fatty acid esters of *N*-(2-hydroxyethyl)-2-pyrrolidone					Lambert et al. (1993)
Cyclic amides: 1-dodecylazacycloheptane-2-one(Azone), 1-geranylazacycloheptan-2-one, 1-farnesylazacycloheptan-2-one, 1-geranylgeranylazacycloheptan-2-one, 1-(3,7-dimethyloctyl)azacycloheptan-2-one, 1-(3,7,11-trimethyldodecyl)azacyclohaptan-2-one, 1-geranylazacyclohexane-2-one, 1-geranylazacyclopentan-2,5-dione, 1-farnesylazacyclopentan-2-one	Azone: enhancer effect can be increased by use of cosolvent such as PG	Azone: 1. Affects lipid structure of SC 2. Increases partitioning 3. Increases membrane fluidity	Azone: 5-fluorouracil, antibiotics, glucocorticoids, peptites, clonazepam, albuterol, estradiol, levonorgestrel, HIV protease inhibitor (LB-71148), betahistine, dihydroergotamine	Azone: significant accelerant effects at low concentration (1–5%), can be applied undiluted to skin without significant discomfort, effective for both hydrophilic and hydrophobic drugs	Stoughton and McClure (1983), Okamoto et al. (1988), Zhou et al. (2005)
Hexamethylenelauramide and its derivatives					Mirejovsky and Takruri (1986)
Diethanolamine, triethanolamine					Mollgaard and Hoelgaard (1983)
Fatty acids					
Linear: linoleic(LIA), valeric, heptanoic, pelagonic, caproic, capric(CA), lauric(LAA), myristic, stearic, oleic(OA), caprylic Branched: isovaleric, neopentanoic, neoheptanoic, neononanoic, trimethyl hexanoic, neodecanoic, isostearic		Selective perturbation of the intercellular lipid bilayers OA: decreases the phase transition temperatures of the lipid, increasing motional freedom or fluidity of lipids	Naloxone, mannitol, betamethasone 17-benzoate, hydrocortisone, acyclovir, nitroglycerin OA: galanthamine, estradiol, levonorgestrel CA: buprenorphine, albiterol LAA: buprenorphine, betahistine	Among stearic, oleic, and linoleic acids, maximum enhancement was observed with linoleic acid	Aungst et al. (1986), (1989), Kogan and Garti (2006)

(*continued*)

TABLE 5.2 (continued)
Chemical Penetration Enhancers

Category and Examples	Cosolvent/Vehicle	Mechanism	Examples of Drugs (Ghosh et al., 1997)	Comment	References
Fatty acid esters					
Aliphatic: isopropyl *n*-butyrate, isopropyl *n*-hexanoate, isopropyl *n*-decanoate, isopropyl myristate (IPM), isopropyl palmitate, octyldodecyl myristate Alkyl: ethyl acetate (EA), butyl acetate, methyl acetate, methylvalerate, methylpropionate, diethyl sebacate, ethyl oleate		IPM: direct action on SC, permeating into liposome bilayers, increasing fluidity Aliphatic: increase diffusivity in the SC and the partition coefficient Alkyl: increase lipid fluidity (similar to DMSO)	IPM: galanthamine, ketorolac, chlorpheniramine, dexbrompheniramine, diphenhydramine, theophylline, pilocarpine, verapamil EA: levonorgestrel, 17β-estradiol, hydrocortisone, 5-fluorouracil, nefedipine		Sato et al. (1988), Friend et al. (1989)
Surfactants					
Anionic: sodium laurate, sodium lauryl sulfate, sodium octyl sulfate		Alter the barrier function of SC, allowing removal of water-soluble agents that normally act as plasticizers		Greater damage and permeation enhancement with anionic surfactants than with nonionic surfactants	Chowhan and Pritchard (1978), Gershbein (1979)
Cationic: cetyltrimethylammonium bromide, tetradecyltrimethylammonium bromide, octyltrimethyl ammonium bromide, benzalkonium chloride, octadecyltrimethylammonium chloride, cetylpyridinium chloride, dodecyltrimethylammonium chloride, hexadecyltrimethylammonium chloride	Significant increases in the flux of lidocaine from saturated systems in PG-water mixtures	Adsorb at interfaces and interact with biological membranes, causing damage to skin		Cationic surfactants are more destructive to skin than anionic surfactants	Gershbein (1979), Aoyagi et al. (1990), Tan et al. (1993)
Zwitterionic surfactants: hexadecyl trimethyl ammoniopropane sulfonate, oleyl betaine, cocamidopropyl hydroxysultaine, cocamidopropyl betaine					Zhang and Somasundaran (2006)

Nonionics:					
polyxamer (231,182,184), polysorbate (20,60), brij (30,93,96,99), span (20,40,60,80,85), tween (20,40,60,80), myrj (45,51,52), miglyol 840	Polysorbate 20 and 60 increased lidocaine flux in the presence of PG	Emulsify sebum, enhancing the thermodynamic activity of coefficients of drugs	Tween 80: ketoprofen Polysorbate 20,60: lidocaine		Aungst et al. (1986), Shen et al. (1976), Mahajour et al. (1993)
Bile salts:					
sodium cholate, sodium salts of taurocholic(TC), glycolic, desoxycholic acids			TC: elcatonin and vit D_3, estradiol and vit D_3		Carelli et al. (1993)
Lecithin					Kato et al. (1987)
Terpenes					
Hydrocarbons: D-Limonene, α-pinene, β-carene Alcohols: α-Terpineol, terpinen-4-ol, carvol Ketones: Carvone, pulegone, piperitone, menthone Oxides: Cyclohexene oxide, limonene oxide, α-pinene oxide, cyclopentene oxide, 1,8-cineole Oils: Ylang ylang, anise, chenopodium, eucalyptus		1. Increase diffusivity of drugs within SC due to disruption of intercellular lipid barrier 2. Open new polar pathways within and across the SC	5-Fluorouracil, aspirin, haloperidol	Hydrocarbon terpenoids were least effective; oxides moderately effective; and the alcohols, ketones, and cyclic ethers most effective accelerants of 5-fluorouracil permeation	Williams and Barry (1991), Hori et al. (1991), Lim et al. (2006)
Organic acids					
Salicylic acid and salicylates (including their methyl, ethyl, and propyl glycol derivatives), citric and succinic acid					Sugibayashi et al. (1988)
Cyclodextrins					
2-Hydroxypropyl-β-cyclodextrin (HPβCD), 2,6-dimethyl-β-cyclodextrin (DIMEB)	Higher penetration of liarzole in DIMEB with PG/oleic acid compared to HPβCD	Form inclusion complexes with lipophilic drugs and increase their solubility in aqueous solutions	Liarzole		Uekama et al. (1985), Frijlink et al. (1976)
Proprietary chemical enhancers					Chan (2005)
Alkyl-2-(*N*,*N*-disubstituted amino)-alkanoate ester (NexAct®), 2-(n-nonyl)-1,3-dioxolane (SEPA®)			Ibuprofen, ketoprofen, alprostadil, testoterone		

* SC: stratum corneum.

Source: Barry, B. in *Percutaneous Penetration Enhancers*, CRC Press, Boca Raton, FL, 1995.

Furthermore, Kanikkannan et al. (2000) proposed that based on the chemical structure of PEs (such as chain length, polarity, level of unsaturation, and presence of specific chemical groups such as ketones), the interaction between the SC and PEs may vary, contributing to the different mechanisms in penetration enhancement. A comprehensive understanding of the mechanisms of action and a judicious selection of CPE would be helpful in the successful development of TDD and DDD products.

5.5 U.S. FOOD AND DRUG ADMINISTRATION (FDA)-APPROVED TDD

There has been an increased focus on the potential of transdermal drug delivery as evident from the increase in the number of patents as well as scientific publications on TDD systems. Many drugs have been evaluated for TDD in prototype patches, either *in vitro* permeation studies using mouse, rat, or human skin; or have reached varying stages of clinical testing. Examples are listed in Table 5.2. Despite a wide array of TDD systems undergoing research and development, only a small percentage of the drugs reaches the market successfully due to three limitations: difficulty of penetration through human skin, skin irritation and allergenicity, and clinical need. In addition, it is generally accepted that the best drug candidates for passive adhesive transdermal patches must be nonionic, low molecular weight (less than 500 Da), have adequate solubility in oil and water (log P in the range 1–3), a low melting point (less than 200°C), and are potent (dose is less than 50 mg/day, and ideally less than 10 mg/day) (Finnin and Morgan, 1999; Guy, 1996; Hadgraft, 1998). Given these operating parameters, the number of drug candidates, which fit the criteria may seem low. Nevertheless, with the development of novel technologies, such constraint may be overcome.

Since the introduction of a TDD for scopolamine in 1981, several new products have been introduced. The U.S. TDD market approached $1.2 billion in 2001 and was based on 11 drug molecules: fentanyl, lidocaine, prilocaine, nitroglycerin, estradiol, ethinyl estradiol, norethindrone acetate, testosterone, clonidine, nicotine, and scopolamine (Retail and Provider Perspective, 2001). Barry (2001) reported that 40% of drug delivery candidate products that were under clinical evaluation and 30% of those in preclinical development in the United States were TDD or DDD systems.

Examples of FDA-approved transdermal patches and their applications are in Table 5.3. Despite a plethora of candidate CPEs to choose from, all currently available TDD products adopt skin occlusion as the primary mechanism for penetration enhancement, perhaps due to its simplicity and convenience, and the following effects on SC (Zhai and Maibach, 2001a, 2002): an increase in SC hydration and a reservoir effect in penetration rates of the drug due to hydration, an increase in skin temperature from 32 to 37°C, and

TABLE 5.3
Examples of FDA-Approved Transdermal Patches, Their Applications, and the Mechanisms/Compounds Used for Penetration Enhancement

Drug	Application(s)	Example(s) of Commercially Available Product(s)	Penetration Enhancement Effect and Penetration Enhancers
Scopolamine	Motion sickness	Transderm scop	Occlusive effect
Fentanyl	Moderate to severe chronic pain	Duragesic	Occlusive effect
Lidocaine	Anesthesia	Lidoderm	Occlusive effect, urea, propylene glycol
Prilocaine	Anesthesia	EMLA anesthetic disc	Occlusive effect, polyoxyethylene fatty acid esters
Testosterone	Hormone replacement therapy	Androderm	Occlusive effect, glycerol monooleate
Estradiol/norethindrone acetate	Hormone replacement therapy	Combipatch	Occlusive effect, silicone, oleic acid, dipropylene glycol
Estradiol	Symptomatic relief of postmenopausal symptoms and prevention of osteoporosis	Alora, climera, esclim, vivelle, vivelle-dot	Occlusive effect Climera: fatty acid esters Vivelle: 1,3-butylene glycerol, oleic acid, lecithin, propylene glycol, dipropylene glycol Vivelle-dot: oleyl alcohol, dipropylene glycol
Norelgestromin/ethinyl estradiol	Contraception	Ortho evra	Occlusive effect, lauryl lactate
Nitroglycerin	Angina pectoris	Nitro-dur, nitrodisc, transderm-nitro	Occlusive effect, fatty acid esters
Clonidine	Hypertension	Catapres-TTS	Occlusive effect
Nicotine	Smoking cessation	Nicoderm CQ	Occlusive effect
Methyphenidate	Attention-deficit hyperactive disorder	Daytrana	Occlusive effect
Selegiline	Depression	Emsam	Occlusive effect
Oxybutynin	Urge/urinary incontinence	Oxytrol	Occlusive effect

the prevention of accidental wiping or evaporation (volatile compound) of the applied compound.

5.6 FUTURE TRENDS

The protective function of human SC imposes physicochemical limitations to the type of molecules that can traverse the barrier. As a result, commercially available products based on TDD or DDD have been limited. Various strategies have emerged over the last decade to optimize delivery. Approaches such as the optimization of formulation or drug-carrying vehicle to increase skin permeability do not greatly improve the permeation of macromolecules.

Sufficient data on chemical enhancers is available, so that the Smith textbook (1st and 2nd edition) provides extensive quantitative data (Smith and Maibach, 1995, 2005). Note that of the several dozen proposed enhancers suggested over 4 decades, few new chemical entities have received widescale usage. Dermatotoxicologic intolerance has been a major limitation. Dermatotoxicology 8th edition will provide a detailed explanation of some of the potential hazard with their long term use.

On the contrary, physical or mechanical methods of enhancing delivery have been more promising. Improved delivery has been shown for drugs of differing lipophilicity and molecular weight including proteins, peptides, and oligonucletides using electrical methods (iontophoresis, electroporation), mechanical (abrasion, ablation, perforation), and other energy-related techniques such as ultrasound and needleless injection (Brown et al., 2006).

Another strategy for penetration enhancement is to exploit the synergistic effects offered by combined techniques. Karande et al. (2004) reported the discovery of synergistic combinations of penetration enhancers (SCOPE), which allow permeation of 10 kDa macromolecules with minimal skin irritation using high-throughput screening method. Kogan and Garti (2006) also showed that the combination of several enhancement techniques led to synergetic drug penetration and decrease in skin toxicity. In essence, the possibilities seem endless in the field of TDD and DDD.

5.7 CONCLUSION

TDD would avoid problems associated with the oral route, as well as the inconvenience and pain associated with needle delivery; and has thus competed with oral and injection therapy for the accolade of the innovative research area for drug delivery. Yet there remains a paucity of candidates for TDD or DDD to be marketed. The reasons are twofold: (1) most candidate drug molecules have low permeation rates through the skin to ever reach clinically satisfactory plasma level; (2) risk of skin irritation and allergic contact dermatitis may be increased by skin occlusion (Zhai and Maibach, 2001b, 2002) or the application of potent PEs (Karande et al., 2004). The ideal characteristics of PEs include the following (Pfister et al., 1990):

- Be both pharmacologically and chemically inert
- Be chemically stable
- Have a high degree of potency with specific activity, rapid onset, predictable duration of activity, and reversible effects on skin properties
- Show chemical and physical compatibility with formulation and system components
- Be nonirritant, nonallergenic, nonphototoxic, and noncomedogenic
- Be odorless, tasteless, colorless, cosmetically acceptable, and inexpensive
- Be readily formulated into dermatological preparations, transdermal patches, and skin adhesives
- Have a solubility parameter approximating that of skin (i.e., 10.5 $cal^{1/2}cm^{3/2}$) (Sloan et al., 1986)

Future studies on the mechanisms of penetration enhancement, the metabolic processes of chemicals within the skin, skin toxicity, as well as the development of novel technologies will improve our knowledge on penetration enhancement. While the current TDD and DDD technologies still offer significant potential for growth, next-generation technologies will enable a much broader application of TDD to the biopharmaceutical industry.

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6 Percutaneous Absorption of Complex Chemical Mixtures

Jim E. Riviere

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6.1 INTRODUCTION

The percutaneous absorption of chemicals is most often studied using single chemicals applied to the surface of skin, often in a vehicle when solubilization is necessary. Exposure in most environmental and occupational scenarios occurs to combinations of chemicals. Similarly, most dermatological drugs are dosed in formulations composed of multiple additives. The difference is that in the pharmaceutical sector, components of a formulation are usually added for a specific purpose and their effects on drug absorption have been studied, or at least acknowledged. In environmental and occupational scenarios, the chemicals to which an individual is exposed are a function of their occurrence in the environment. The effect of one chemical, modulating absorption of a second mixture component, is not known. Risk assessments on topical exposure to chemical mixtures are presently an area of intense interest, but few quantitative approaches have been proposed.

The potential for chemical mixture interactions affecting systemic drug and chemical disposition and toxicity has been well recognized for many years and has been comprehensively reviewed elsewhere (Bliss, 1939; Yang, 1994; Pohl et al., 1997; Haddad et al., 2000, 2001; Borgert et al., 2001; Groten et al., 2001; Feron and Groten, 2002; Walker et al., 2005; Mumtaz et al., 2006). Similarly, the potential for drug–drug pharmacokinetic interactions has been recently recognized and debated in the context of the development of cassette dosing in drug discovery screening (White and Manitpisitkul, 2001; Christ, 2001; Singh, 2006). Despite this widespread acknowledgment of the importance of chemical interactions in systemic pharmacology and toxicology, little attention outside of the dermatological formulation arena has been paid to interactions that may occur after topical exposure to complex mixtures. The focus of this chapter is to provide a brief review into factors that should be considered when chemical mixtures are topically applied to skin.

6.2 LEVELS OF INTERACTION

The focus on any interaction is usually related to a compound of pharmacological or toxicological interest. The concern is then on how other chemicals in the mixture modulate the percutaneous absorption or dermatotoxicity of this chemical of interest. To clarify this discussion, we will refer to the compound of pharmacological or toxicological interest as the "marker" compound and all other substances present in the mixture as "components." In a simple binary mixture, the component would be the vehicle. It must be stressed that the purpose of selecting a component of a mixture as a marker compound does not confer special importance to this chemical relative to the other components present. It is purely an artificial construct to provide a frame of reference on which chemical interactions can be discussed. In many cases, multiple components may in fact be toxic or cause irritation.

Previously, we have presented a conceptual framework upon which the study of chemical interactions involved in compound percutaneous absorption can be based, termed mechanistically defined chemical mixtures (MDCM) (Baynes et al., 1996; Qiao et al., 1996; Williams et al., 1996). This approach assumes that components that are capable of modulating a marker's absorption or cutaneous disposition would result in an altered pharmacological or toxicological effect. Table 6.1 lists a series of levels in which chemical and biological interactions could occur.

The first potential for chemical–chemical interactions is on the surface of the skin. The types of phenomena that could occur are governed by the laws of solution chemistry, and include factors such as altered solubility, precipitation, supersaturation, solvation, or volatility; as well as physical-chemical effects such as altered surface tension from the presence of surfactants, changed solution viscosity and micelle formation (Idson, 1983; Williams and Barry, 1998; Barry, 2001; Moser et al., 2001; van der Merwe and Riviere, 2005). For some of these effects, chemicals act

TABLE 6.1
Levels of Potential Interactions after Topical Exposure to Chemical Mixtures

Surface	Chemical–chemical (binding, ion-pair formation, etc.)
	Altered physical-chemical properties (e.g., solubility, volatility, critical micelle concentration)
	Altered rates of surface evaporation
	Occlusive behavior
	Binding or interaction with adnexial structures or their products (e.g., hair, sweat, sebum)
Stratum Corneum	Altered permeability through lipid pathway (e.g., enhancer)
	Altered partitioning into stratum corneum
	Extraction of intercellular lipids
Epidermis	Altered biotransformation
	Induction or modulation of inflammatory mediators
Dermis	Altered vascular function (direct or secondary to mediator release)

independent of one another. However, for many the presence of other component chemicals may modulate the effect seen. Chemical interactions may further be modulated by interaction with adnexial structures or their products such as hair, sebum, or sweat secretions. The result is that when a marker chemical is dosed on the skin as a component of a chemical mixture, the amount freely available for subsequent absorption may be significantly affected. The primary driving force for chemical absorption in skin is passive diffusion that requires a concentration gradient of thermodynamically active (free) chemical. We have developed an *in vitro* membrane coated fiber (MCF) approach to quantitate the physical-chemical interactions seen in dosing solutions (Xia et al., 2005, 2006) that has promise to quantitatively assess the impact of such interactions on partitioning into skin.

The next level of potential interaction are those involving the marker or component chemicals with the constituents of the stratum corneum. These include the classic enhancers such as oleic acid, Azone®, or ethanol, widely reviewed elsewhere (Williams and Barry, 1998). These chemicals alter a compound's permeability within the intercellular lipids of the stratum corneum. Similarly, the partition coefficient between the drug in the surface dosing vehicle and stratum corneum lipids may be altered if chemical components of the mixture also partition and diffuse into the lipids and thus alter their composition. Organic vehicles on the surface of the skin may extract stratum corneum lipids that would alter permeability to the marker chemical (Monteiro-Riviere et al., 2001; Rastogi and Singh, 2001), a phenomenon seen with repeated topical exposure to jet fuels (Muhammad et al., 2005). Compounds may also bind to stratum corneum constituents forming a depot.

The next level of interaction would be with the viable epidermis. The most obvious point of potential interaction would be with a compound that undergoes biotransformation (Bronaugh et al., 1989; Mukhtar, 1992). A marker and component could interact in a number of ways, including competitive or noncompetitive inhibition for occupancy at the enzyme's active site, or induction or inhibition of drug metabolizing enzymes. Other structural and functional enzymes could also be affected (e.g., lipid synthesis enzymes), which would modify barrier function (Elias and Feingold, 1992). A penetrating marker or component could also induce keratinocytes to release cytokines or other inflammatory mediators (Luger and Schwarz, 1990; Allen et al., 2000; Monteiro-Riviere et al., 2002), which could ultimately alter barrier function in the stratum corneum or vascular function in the dermis. Alternatively, cytokines may modulate biotransformation enzyme activities (Morgan, 2001). A dermatopharmacokinetic scheme taking into account marker and a single-component (vehicle) compound penetration and potential interaction is depicted in Figure 40.7 of Chapter 40 of this book.

The final level of potential interaction is in the dermis where a component chemical may directly or indirectly (e.g., via cytokine release in the epidermis) modulate vascular uptake of the penetrated marker (Riviere and Williams, 1992; Williams and Riviere, 1993; Cross and Roberts, 2006). In addition to modulating transdermal flux of chemical, such vascular modulation could also affect the depth and extent of marker penetration into underlying tissues.

It must be stressed that interactions at all of these levels could occur simultaneously, and multiple components could be affecting marker penetration, as well as other component disposition in skin. These can optimally be teased apart using a hierarchy of experimental model systems, which are only responsive to specific levels of interactions. A scheme used in our laboratory to study these effects is depicted in Table 6.2. The important point to stress about this scheme is that when a chemical mixture absorption is being assessed, the biological complexity of the experimental model system must be sufficient to detect the interaction. It is the sum of all interactions that ultimately determines the mixture's effect on marker absorption or skin disposition.

6.3 EXAMPLES OF MIXTURE INTERACTIONS

The above conceptual framework for assessing the importance of chemical mixture interactions on a marker chemical absorption or cutaneous disposition is by no way unique.

TABLE 6.2
Experimental Model Systems Used to Assess Levels of Interactions

In Vitro Physical-Chemical Determinations	Solubility
	Viscosity
	Critical micelle concentration
	Partition coefficients
In Vitro Silicone Membrane Diffusion Cells or Membrane Coated Fibers (MCF)	*Above effects*
	Assess partitioning and diffusion through defined membrane
In Vitro Dermatomed Skin Diffusion Cells[a]	*Above effects*
	Partitioning and diffusion parameters through stratum corneum and viable epidermis
	Epidermal biotransformation
Ex Vivo Isolated Perfused Porcine Skin Flaps (IPPSF)	*Above effects*
	Vascular modulation
	Inflammatory mediators
	Dermal binding and metabolism
In Vivo Whole Animals or Humans	*All above effects*
	Systemic feedback operative

[a] Human and porcine skin (correlated to IPPSF and *in vivo*).

As mentioned earlier, many of these interactions have been defined in binary mixtures consisting of marker chemical and vehicle, and thus are often categorized as vehicle effects. The problems occur when the absorption of complex mixtures, such as environmental contaminants at waste sites (≈50) or hydrocarbon fuels (>200) are considered. Many "simple" occupational mixtures may contain upward of 5–10 compounds. Some interactions may be synergistic, others antagonistic. The result observed is a vectorial sum of all interactions. The principles of complexity and chaotic systems teach us that when simple systems are added together, emergent behavior may occur, which is not predictable from examining simpler systems with fewer components (Bar-Yum, 1997). We have demonstrated this lack of predictability when the behavior of single and 2×2 combinations of jet fuel additives did not predict the behavior of hydrocarbon marker absorption when all three additives were present (Baynes et al., 2001).

A number of investigators have probed nonvehicle type mixture effects on topical absorption. Reifenrath and coworkers (1996) demonstrated that exposure of skin to a complex chemical irritant (hydroxylammonium nitrate, triethanolammonium nitrate, water) resulted in enhanced skin permeability of subsequently applied benzoic acid using *in vitro* and *in vivo* models. Based on their own data and an interpretation of other irritant studies (Bronaugh and Stewart, 1985; Wilhelm et al., 1991), they concluded that *in vitro* and *in vivo* studies agree when alterations occur to the stratum corneum barrier. However, when irritants influence other aspects of cutaneous physiology (e.g., vesication, erythema), then the *in vivo* response may be exaggerated. A role of estradiol in modulating phenol absorption has been reported (Abou-Hadeed et al., 1998).

Our laboratory initially implemented the MDCM approach studying the effects of sodium lauryl sulfate (surfactant), methyl nicotinate (rubefacient), and stannous chloride (reducing agent) in aqueous mixtures containing either acetone or DMSO on the percutaneous absorption of parathion (Qiao et al., 1996) and benzidine (Baynes et al., 1996). These studies in parathion mixtures containing up to six components demonstrated significant modulation (11-fold) of marker compound absorption and skin penetration depending on the composition of the dosed mixture. Higher-level statistical interactions between mixture components were detected. Benzidine absorptions were modulated 10-fold. In both studies, some compounds tended to enhance absorption (sodium lauryl sulfate, DMSO) while others (stannous chloride) tended to retard absorption when present in a mixture. Methyl nicotinate blunted parathion absorptive flux and changed the shape of the absorption profile. The effects of some mixture components were most dramatically evidenced by changes in the absorption/skin deposition ratios. Stratum corneum barrier function as measured by transepidermal water loss in the benzidine study changed as a function of mixture composition (Baynes et al., 1997). These studies concluded that the percutaneous absorption and skin deposition of the marker compounds benzidine and parathion were significantly dependent upon the composition of the chemical mixture in which they were dosed. Compass plots (Figure 6.1), a novel graphical tool was developed to statistically evaluate and illustrate the interactions present in such complex mixtures (Budsaba et al., 2000).

Similar mixture interactions were detected in a study of pentachlorophenol (PCP) percutaneous absorption (Riviere et al., 2001), where PCP flux varied 12-fold depending on the mixture applied. These data are illustrated in Figure 6.2. In contrast, absorption of 3,3′,4,4′-tetrachlorobiphenyl (TCB) and 3,3′,4,4′,5-pentacholrobiphenyl (PCB) were minimal in this system under all exposure scenarios, again stressing the

central role that the individual chemical's properties have on their susceptibility to mixture interactions. A recent study clearly showed that the dermal absorption of the biocide triazine was enhanced when nickel or a nitrosamine (NDELA) was present in either a mineral oil- or synthetic aqueous propylene glycol–based cutting fluid (Baynes et al., 2005a), or when skin was preexposed to trichloroethylene (Baynes et al., 2005b).

Percutaneous absorption of topically applied permethrin and *N*,*N*-diethyl-*m*-toluamide (DEET), two chemical entities putatively involved in the Gulf War syndrome, was modulated by coexposure to one another, as well as simultaneous exposure to topical jet fuels, sulfur mustard or organophosphates (Baynes et al., 2002; Riviere et al., 2002a,b). The highest flux of labeled permethrin was observed when pyridostigmine and the organophosphate nerve agent simulant DFP were infused into the *in vitro* and *ex vivo* models, an experimental manipulation, which also blunted inflammatory mediator release simultaneously assessed (Monteiro-Riviere et al., 2003). This later observation highlights the importance of both topical and systemic chemical exposures to modulate absorption of marker compounds.

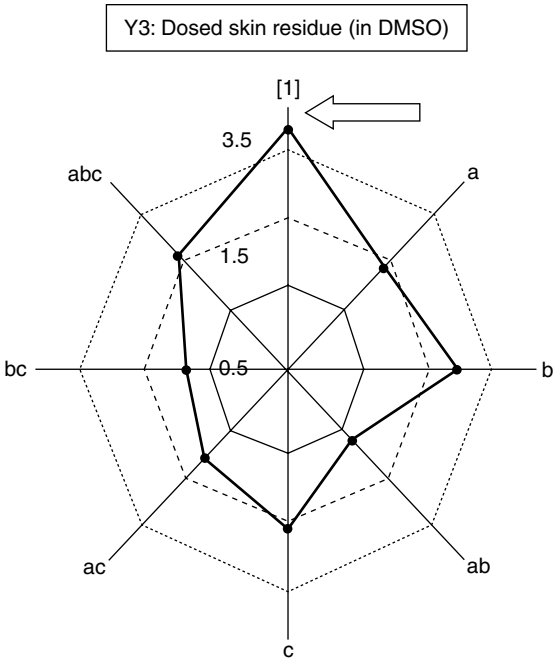


FIGURE 6.1 Compass plot illustrating a three-component chemical interaction. Mean (---) and confidence intervals (—) are depicted for all combinations of treatments a, b, and c. Points outside of this polygon (⇒) are significantly different.

6.4 A MODIFIED QSAR APPROACH TO QUANTITATE MIXTURE INTERACTIONS

Although studies such as those briefly reviewed above clearly demonstrate that mixture interactions occur, how does one quantitatively predict their impact on compound absorption? Significant progress has been made in predicting the dermal absorption or penetration of topically applied chemicals across skin using quantitative structure activity relationship (QSAR) models based on linear-free energy relations (LFER) that link penetrant molecular descriptors to skin permeability (k_p) (Potts and Guy, 1992; Abraham et al., 1999; Geinoz et al., 2004; Cronin, 2006). These efforts have employed chemicals applied to the surface of skin in

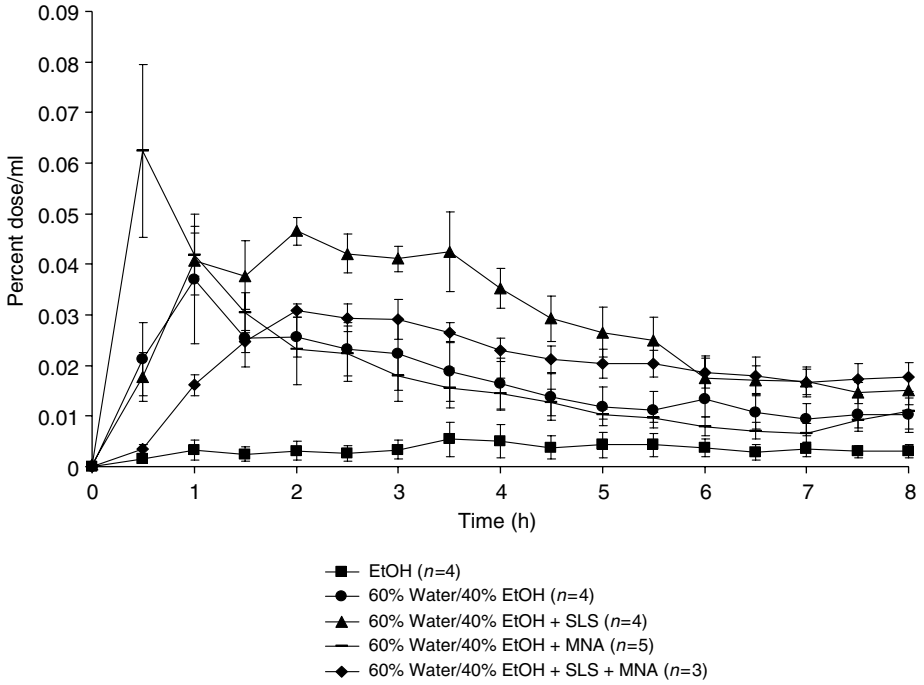


FIGURE 6.2 IPPSF perfusate absorption profiles of PCP (mean ± SEM).

single solvent or aqueous systems. The effects of mixture interactions have never been quantitatively addressed in the framework of QSPR models.

We have recently developed a hybrid LFER-based QSAR approach to predict the effect of mixture components on their absorption though porcine skin (Riviere and Brooks, 2005, 2007) based on the molecular descriptors for the chemical, modified by a mixture factor (MF), which accounts for the physicochemical properties of the vehicle/mixture components. We employed Abraham's LFER model as our base equation since it is representative of the dermal QSPR approaches presently available. Studies were conducted using *in vitro* porcine skin diffusion cells (Riviere and Brooks, 2005). The basic Abraham LFER model is

$$\log k_p = c + a\sum \alpha^H{}_2 + b\sum \beta^H{}_2 + s\pi^H{}_2 + rR_2 + vV_x \qquad (6.1)$$

where

k_p = Permeability constant for the diffusion cell experiments
$\Sigma\alpha^H{}_2$ = Hydrogen-bond donor acidity
$\Sigma\beta^H{}_2$ = Hydrogen-bond acceptor basicity
$\pi^H{}_2$ = Dipolarity/polarizability
R_2 = Excess molar refractivity
V_x = McGowan volume

To incorporate mixture effects, another term is added called the mixture factor (MF) yielding:

$$\log k_p = c + m\mathrm{MF} + a\sum \alpha^H{}_2 + b\sum \beta^H{}_2 + s\pi^H{}_2 + rR_2 + vV_x \qquad (6.2)$$

The parameters *c*, *m*, *a*, *b*, *s*, *r*, and *v* are strength coefficients coupling the molecular descriptors to skin permeability in the specific experimental system. The value for the MF is determined by examining the residual plot (actual – predicted log k_p) generated from Equation 6.2 based on molecular descriptors of the permeants as a function of the physical-chemical properties of the mixture/solvents in which they were dosed.

A large data set of 12 compounds in 24 mixtures were employed for a total of 288 treatment combinations. These data were previously analyzed and grouped into penetrants that were differentially modified by solvent–mixture combinations (van der Merwe and Riviere, 2006), an exercise which suggested the types of physical-chemical properties of the mixture components to analyze, including parameters of molecular size and volume, hydrogen bonding, p*K*a, Henry's law constant, polarizability, refractive indices, etc. We then computed a composite physical-chemical MF by weighting the component's physical-chemical parameter (e.g., refractive index) by its contribution to its MF based on the summation of the weight percentage of each of the bulk components in the mixtures for a particular parameter. Minor mixture components based on weight percentages, in this case the actual penetrant, did not materially contribute to the value of the MF for a specific treatment and could be excluded. To confirm selection of these specific parameters, principal component analyses of these descriptor's effects on k_p also yielded three groups of descriptors, which accounted for different patterns of variability seen.

Figure 6.3 depicts the predicted versus observed compound fluxes without (Figure 6.3A) and with (Figure 6.3B) a mixture factor included. These data clearly demonstrate the feasibility of using this approach to quantitatively gauge the impact of a mixture on compound absorption.

6.5 CONCLUSIONS

This brief review clearly demonstrates that dermal absorption of a chemical administered in a complex chemical mixture of even three to four components, cannot be easily predicted from simpler exposure scenarios unless the properties of the mixtures are included. In some cases, certain additives have consistent effects across mixtures (e.g., sodium lauryl sulfate, stannous chloride) due to a consistent mechanism of action. One approach to predict the effect of a complex mixture is to define the components based on their potential mechanism of action relative to modulating absorption. Use of multiple levels of experimental models facilitates this endeavor. Precise definition and quantitation of such interactions would allow for the development of complex interactive dermatopharmacokinetic models (Williams et al., 1996). However, the picture is not as clear as this conclusion implies. The effect of components on marker absorption is dependent on the chemical properties of the marker being studied, thus PCP behaved differently than PCB or TCB. Similarly, component effects on parathion and benzidine were different, an observation that is quantitated by the MF in the hybrid QSAR approach.

It is theoretically feasible that some mixture components, such as classical enhancers like oleic acid, might induce what amounts to a phase-transition in the stratum corneum lipids that would totally change the types of interactions seen with other mixture components. This would be an example of emergent behavior, a phenomenon that would make extrapolations from experimental studies or simpler mixture exposures problematic. Evidence of this was seen in the parathion mixture experiments where certain combination resulted in a great enhancement of treatment variance, an indicator that the system was no longer stable.

The risk assessment of topical chemical mixtures is a research and toxicological exposure paradigm that will become increasingly important as occupational and environmental problems become more common. Interactions should be defined in specific physical-chemical and biological terms so that methods to integrate findings across studies can be developed. Model systems should not be overinterpreted, and caution must be exercised when extrapolating from simple *in vitro* models up the ladder of biological complexity. The use of mathematical models is a powerful tool to link these studies across chemicals, mixtures, and experimental systems.

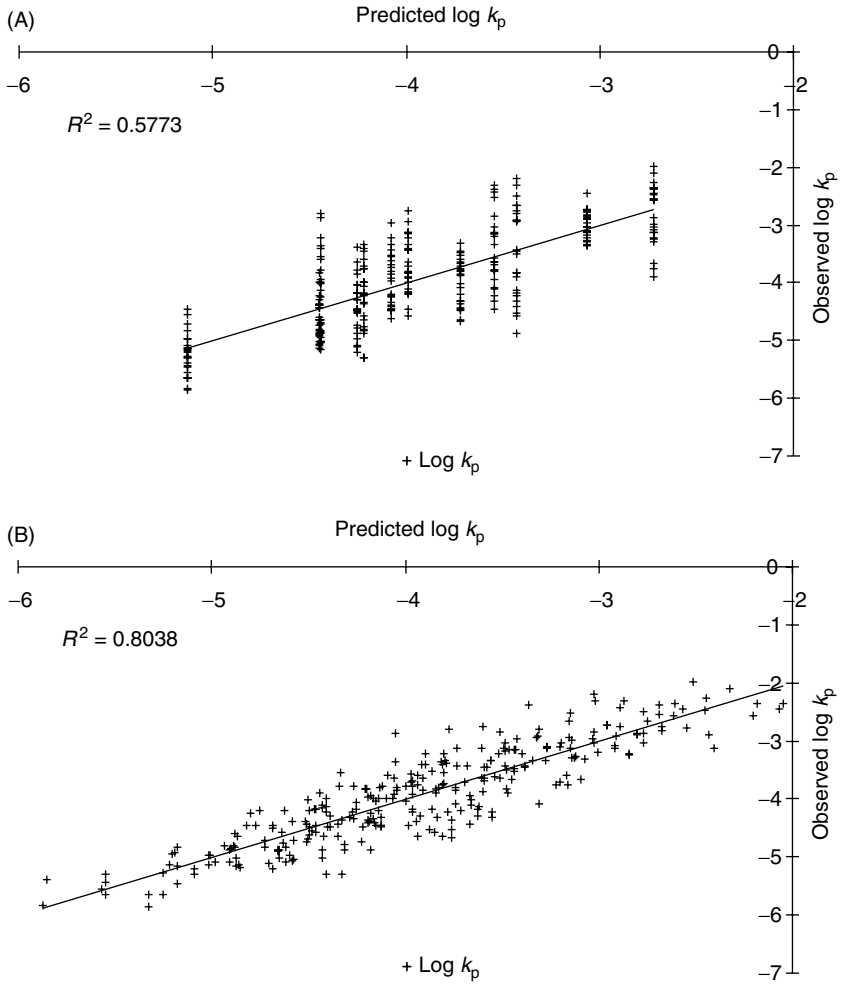


FIGURE 6.3 Predicted versus observed log k_p of chemical absorption in *in vitro* porcine skin diffusion cells. (A) no mixture factor. (B) Mixture factor equals refractive index.

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7 Percutaneous Absorption: Short-Term Exposure, Lag Time, Multiple Exposures, Model Variations, and Absorption from Clothing

Ronald C. Wester and Howard I. Maibach

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7.1 INTRODUCTION

The area of percutaneous absorption has been established as a significant part of dermatotoxicology. Human health risk assessment includes an estimate for percutaneous absorption where dermal exposure is involved. Some estimate of percent dose absorbed or steady-state absorption (flux) is included. Behind these generated numbers lies the question of validation. First, human exposure is a risk end point, and if a model is used, that model should be validated for humans *in vivo*. Second, there is the question of relevance of the particular risk assessment situation to the provided percutaneous absorption data. For example, is an absorption estimate derived over a long period of exposure applicable to a short exposure period? (There are some examples where this is not the case.) Also, multiple exposures (daily or weekly) can exceed single exposure estimates in some situations. Third, some limitations (lag time, lipophilicity) of the *in vitro* diffusion model are shown. Finally, the data showing skin delivery and percutaneous absorption of chemicals from clothing fabric are discussed. The overall interest is relevant and validates percutaneous absorption data and proper data interpretation.

7.2 SHORT-TERM EXPOSURE TO HAZARDOUS CHEMICALS

Exposure to hazardous chemicals in water during a bath or swim is on the order of 30 min to an hour. Some tasks at work or at home where exposure may occur can be of the same length of time. A hazardous spill is usually washed with soap and water within this time frame. The standard workday is 8 h, punctuated with breaks during this time period. Assessment of skin absorption in the laboratory is usually in the magnitude of 24 h, or some steady-state rate achieved in the course of 24+ h of exposure. Linearity in skin absorption is assumed, and the appropriate calculations to the desired time period are made.

Table 7.1 shows that the *in vivo* percutaneous absorption for a 24-h exposure is 51% for a dose of benzo[a]pyrene and 18.9% for a dose of DDT. To simulate short-term exposure,

TABLE 7.1
Short-Term Wash Recovery for Benzo[a]pyrene and DDT: 25-min Exposure versus 24-h Exposure

	Percentage Dose		
	Short Exposure (25 min), *In Vitro*		
Chemical	**Receptor Fluid**	**Skin**	**Long Exposure (24 h), *In Vivo***
Benzo[a]pyrene	0.00 ± 0.00	5.1 ± 2.1	51.0 ± 22.0
DDT	0.00 ± 0.00	16.7 ± 13.2	18.9 ± 9.4

Note: The chemical in acetone vehicle was dosed on human skin *in vitro*, then washed with soap and water after a 25-min period. The *in vivo* studies were a 24-h exposure with acetone vehicle dosing.

TABLE 7.2
Exposure of Human Skin to Cadmium in Water for 30 min Followed by Skin Surface Wash with Soap and Water and Then 48-h Perfusion with Human Plasma

	Percentage Dose	
Treatment	**Skin Content**	**Plasma Receptor Fluid**
30-min exposure only	2.3 ± 3.3	0.0 ± 0.0
30-min exposure followed by 48-h perfusion	2.7 ± 2.2	0.6 ± 0.8
Statistics	$p = .77$	$p = .04$[a]

Note: $n = 9$ (3 human skin sources × 3 replicates each). This study simulates a 30-min exposure of human skin to cadmium in water (swim, bath) followed by a soap-and-water surface wash. Cadmium is able to bind to human skin in the 30-min exposure time and then be absorbed into the body during the remainder of the day.

[a] Statistically significant difference.

TABLE 7.3
Effect of Exposure Time on Mercury Percutaneous Absorption *In Vitro* in Human Skin

	Percentage Dose	
Time/Treatment	**Receptor Fluid**	**Skin Content**
30 min only ($n = 9$)	0.01 ± 0.00[a]	5.5 ± 5.2[a,b]
30 min followed by 48-h perfusion ($n = 9$)	0.09 ± 0.05[a,b]	6.3 ± 4.9[a,b]
24 h ($n = 6$)	0.06 ± 0.03[b]	35.4 ± 15.2[a]

[a] Significant difference ($p < .05$ or greater).

[b] Nonsignificant difference.

human skin was dosed and the skin surface washed with soap and water after 25 min of dosing. The receptor fluid contained, as expected, no chemical. However, the skin was assayed and benzo[a]pyrene levels were at 5.1% and DDT levels were at 16.7% (the same as for 24-h exposure). In the short 25-min exposure, sufficient chemical had partitioned from the skin surface into the interior, or was so bound that soap and water wash did not remove the chemical (Wester et al., 1990).

In the course of studying cadmium skin absorption, short-term exposure to human skin *in vitro* was examined. This study simulated a 30-min exposure of human skin to cadmium in water (swim, bath) followed by a soap-and-water surface wash. Table 7.2 shows that with 30-min exposure alone, receptor fluid (human plasma) contained no cadmium (0.0%) but that skin content was 2.3% of the dose. To determine if cadmium would migrate from the skin into the plasma receptor fluid (and thus be a systemically absorbed chemical), some skin samples were further perfused for an additional 48 h. Some cadmium in the skin did migrate into the plasma perfusate (0.6%; statistically significant at $p = .04$) (Wester et al., 1992b).

Table 7.3 shows the effect of exposure time on mercury ($HgCl_2$) in water as percutaneous absorption *in vitro* in human skin. With 24 h of exposure, receptor fluid accumulation was 0.06%; however, skin content was 35.4%. Human skin has great attraction for mercury in water. Similar to the study just cited, mercury was applied to human skin for 30 min and the skin was washed with soap and water. Mercury was at low levels in receptor fluid (0.01%) but skin content was a robust 5.5%. Continued perfusion increased receptor fluid content to 0.09% (statistically significant difference $p < .05$). In other words, the mercury quickly partitioned into human skin, and then was slowly absorbed into the perfusate (body).

In each of the cases cited (DDT, benzo[a]pyrene, cadmium, mercury), the chemical exhibited a capacity to quickly partition into human skin with a short-term exposure of 30 min.

7.3 LAG TIME

There is a calculation from *in vitro* diffusion studies called the lag time. A line is drawn along an area of steady-state absorption to the abscissa (horizontal sequence of time) and the intercept with the abscissa is the lag time. This *in vitro* diffusion parameter should not be confused with the actual time that a chemical takes for percutaneous absorption *in vivo*. Table 7.4 gives the flux and lag time for hydroquinone *in vitro* percutaneous absorption in human skin dosed with a 2% hydroquinone cream. The lag time is 8 h. The same

TABLE 7.4
Parameters of *In Vitro* Hydroquinone Percutaneous Absorption in Human Skin

Treatment	Flux (μg/h cm²)	Lag Time (h)
HQ cream	2.94	7.99
HQ cream + inhibitor	2.93	8.03

Note: *In vivo* in human volunteers, hydroquinone is readily detected in blood within 30 min following topical application. The 8-h *in vitro* diffusion lag time has no relevance to the actual *in vivo* percutaneous absorption.

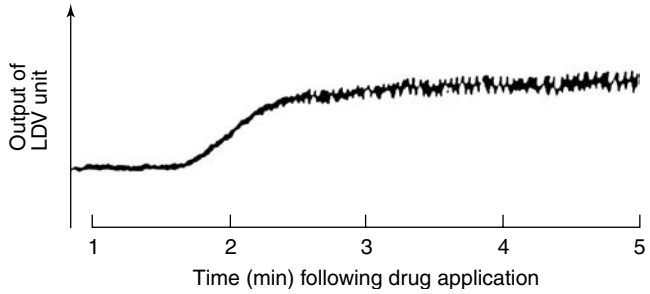


FIGURE 7.1 Laser Doppler velocimetry (LDV) measures human skin blood flow *in vivo*. Methyl nicotinate was able to penetrate and cause a pharmacological reaction in 2 min. This shows that *in vivo* percutaneous absorption can be rapid.

hydroquinone dose/vehicle was topically applied to human volunteers and hydroquinone was detected in blood within 30 min. Therefore, the 8-h *in vitro* diffusion lag time has no relevance to actual *in vivo* percutaneous absorption. The lag time is simply an artificial derivation of the *in vitro* diffusion system.

Laser Doppler velocimetry (LDV) is able to detect changes in human skin blood flow. Topical application of methyl nicotinate (a vasodilator) in human volunteers causes changes in skin blood flow within 2 min (Wester and Maibach, 1984) (Figure 7.1).

The end point for *in vivo* percutaneous absorption is the blood (systemic) in the microcirculation in the upper dermis at the epidermal junction. *In vitro* diffusion adds some dermis and solubility and detection sensitivity limits the process.

7.4 MULTIPLE EXPOSURES IN THE SAME DAY

On a historic and empiric basis, topical applications of hydrocortisone and other corticosteroids frequently use repeated, rather than single, bolus applications of drug to the skin. It is commonly assumed that multiple applications of hydrocortisone effectively increase its bioavailability and absorption. A long-term, multiple-dose rhesus monkey study by Wester et al. (1980) indicated that this was true. However, short-term experiments in the rhesus monkey by Wester et al. (1977) and long-term pharmacokinetic assays by Bucks et al. (1985) did not show an increase in hydrocortisone absorption following multiple dosing. An investigation was designed to determine if multiple-dose therapy (dosing the same site three times in the same day) would increase drug bioavailability in human skin. The study was done *in vivo* using male volunteers (from whom informed consent had been obtained) and *in vitro* with human skin. Hydrocortisone was in either a solvent (acetone) vehicle or in a cream base vehicle.

In each *in vivo* procedure in this crossover study, the subjects were healthy male volunteers, 25–85 years of age, from whom informed consent has been obtained. The treatments were performed on two adjacent sites on each forearm. Each site received a different treatment; each was performed 2–3 weeks apart, alternating forearms between the treatments to allow for systemic and dermal clearance of residual hydrocortisone and radioactivity. The dosing sequence was as follows:

Treatment	Dose per Application (μg/cm²)	Cumulative Dose (μg/cm²)	Total Vehicle Volume (μL)	
			Acetone	Cream
1[a]	13.33	13.33	20	100
2[b]	40.00	40.00	20	100
3[c]	13.33	40.00	60	100

[a] Single dose of 13.33 μg/cm², administered in 20 μL of vehicle.
[b] Single dose of 40.0 μg/cm², administered in 20 μL of vehicle.
[c] Three serial 13.33 μg/cm² doses each administered in 20 μL of vehicle (total 60 μg).

In vitro, three separate human donor skin sources with replicates per each experiment were used. Small cells were of the flow-through design with 1 cm² surface area. Buffered saline at a rate of 1.25 mL/h (1 reservoir volume) served as a receptor fluid. Human cadaver skin was dermatomed to 500 μm and stored refrigerated at 4ºC in Eagle's minimum essential medium. The skin was used within 5 days. This preservation/use regimen follows that used by the human skin transplant bank. Radiolabeled hydrocortisone in either acetone or cream base vehicle was applied to the skin per the study design.

Table 7.5 shows that *in vivo*, the multiple dose (×3) significantly increased hydrocortisone percutaneous absorption for acetone vehicle ($p < .05$) and for the cream vehicle ($p < .006$). Statistically, *in vitro* (Table 7.6), there was no difference with multiple dose; however, the trend was the same as with *in vivo*. Figures 7.2 and 7.3 show the enhanced absorption *in vivo* from the acetone vehicle and the cream vehicle.

This study suggests that triple therapy in humans may have some advantage (Melendres et al., 1992). If increased bioavailability is desired, then multiple-application therapy may be the answer, if patient convenience is not an issue. Our data suggest the possibility that increased bioavailability is related to reapplication of vehicle; hence, a case may be made for increasing hydrocortisone bioavailability merely by applying serial doses of vehicle to a previously applied single dose of hydrocortisone at the skin surface. Such an experiment would verify if the solvent–vehicle effect was the only component by which multiple application of hydrocortisone

TABLE 7.5
Predicted and Observed Hydrocortisone Absorption: *In Vivo*

		Hydrocortisone Absorbed (μg/cm²)	
Vehicle	**Dosing Sequence**	**Predicted**	**Observed**
Acetone[a]	13.3 μg/cm² × 1	–	0.056 ± 0.073
	40.0 μg/cm² × 1	0.168[e]	0.140 ± 0.136[c]
	13.3 μg/cm² × 1	0.168	0.372 ± 0.304[c]
Cream[b]	13.3 μg/cm² × 1	–	0.31 ± 0.43
	40.0 μg/cm² × 1	0.93[e]	0.91 ± 1.55[d]
	13.3 μg/cm² × 1	0.93	1.74 ± 0.93[d]

Note: Different volunteers were used for each formulation; therefore, comparison of absolute bioavailability across vehicle is not justified.

[a] $n = 6$; mean ± SD.

[b] $n = 5$; mean ± SD.

[c] Statistically different ($p < .05$), paired *t*-test.

[d] Statistically different ($p < .006$), paired *t*-test.

[e] 0.168 μg/cm² is 3 × the measured value of 0.056 μg/cm² is 3 × the measured value of 0.31 μg/cm².

TABLE 7.6
Predicted and Observed Hydrocortisone Absorption: *In Vitro*

		Hydrocortisone (μg/cm²)			
		Receptor Fluid		Skin	
Vehicle	**Dosing Sequence**	**Predicted**	**Observed**	**Predicted**	**Observed**
Acetone[a]	13.3 μg/cm² × 1	–	0.13 ± 0.05	–	0.87 ± 0.23
	40.0 μg/cm² × 1	0.39[b]	0.35 ± 0.22	2.61[b]	2.21 ± 2.05
	13.3 μg/cm² × 1	0.39	0.55 ± 0.75	2.61	2.84 ± 2.05
Cream[a]	13.3 μg/cm² × 1	–	0.53 ± 0.029	–	0.30 ± 0.24
	40.0 μg/cm² × 1	0.16[b]	0.23 ± 0.03	0.90[b]	0.86 ± 0.53
	13.3 μg/cm² × 3	0.16	0.27 ± 0.21	0.90	1.19 ± 0.43

[a] $n = 3$; mean ± SD.

[b] 0.39 μg/cm² is 3 × the measured value of 0.13 μg/cm²; 0.16 μg/cm² is 3 × the measured value of 0.053 μg/cm²; 2.61 μg/cm² is 3 × the measured value of 0.87 μg/cm²; 0.91 μg/cm² is 3 × the measured value of 0.30 μg/cm².

in acetone increased its bioavailability in human skin. The cream vehicle was equal in amount for each treatment. Reapplication of cream in triple therapy may have "activated" any hydrocortisone bound in the stratum corneum reservoir.

From a toxicological viewpoint, a question remains as to whether multiple exposures during the day will differ from a single continuous exposure. Also, will varying conditions affect absorption (sweat or rainfall "activating" the absorption system as suggested by increased vehicle situation)?

7.5 MULTIPLE DOSING: AZONE SELF-ENHANCED PERCUTANEOUS ABSORPTION

Azone (1-dodecylazacycloheptan-2-one) is an agent that has been shown to enhance percutaneous absorption of drugs. Azone is thought to act by partitioning into skin lipid bilayers and thereby disrupting the structure. An open-label study was done with nine volunteers (two males, seven females; aged 51–76 years) in whom Azone cream (1.6%; 100 mg) was topically dosed on a 5 × 10 cm area of the ventral forearm for 21 consecutive days. On day 1, 8, and 15, the Azone cream contained 47 μCi of [^{14}C] Azone. The skin application site was washed with soap and water after each 24-h dosing. Percutaneous absorption was determined by urinary radioactivity excretion. The [^{14}C] Azone was ring labeled [^{14}C]-2-cycloheptan. Radiochemical purity was >98.6% and cold Azone purity was 99%. Percutaneous absorption of the first dose (day 1) was 1.84 ± 1.56% (SD) of applied amount for 24-h skin application time. Day 8 percutaneous absorption, after repeated application, increased significantly ($p < .002$) to 2.76 ± 1.91%. Day 15 percutaneous absorption after continued repeated application stayed the same at

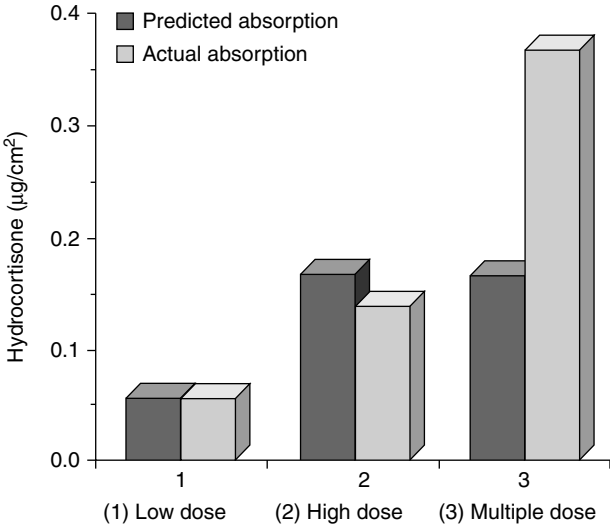


FIGURE 7.2 Hydrocortisone *in vivo* percutaneous absorption in human with acetone vehicle and single and multiple dosing. The multiple dosing (triple therapy) exceeded predicted absorption and was statistically ($p < .006$) greater than the single high dose.

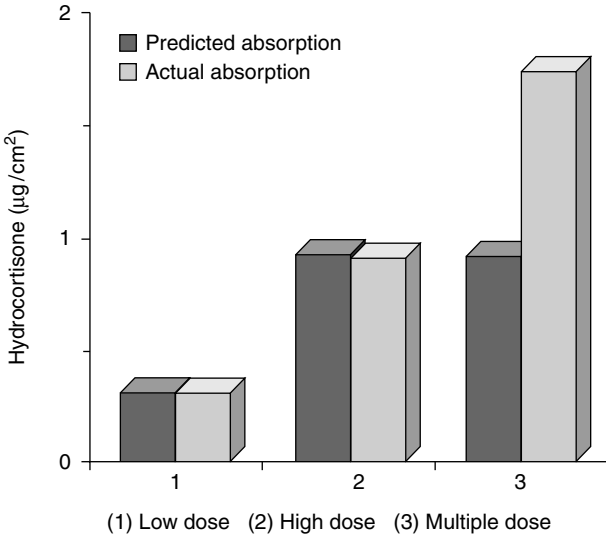


FIGURE 7.3 Hydrocortisone *in vivo* percutaneous absorption in human with cream vehicle and single and multiple dosing. The multiple dosing (triple therapy) exceeded predicted absorption and was statistically ($p < .006$) greater than the single high dose.

$2.72 \pm 1.21\%$. In humans, repeated application of Azone results in an initial self-absorption enhancement, probably due to its mechanism of action. However, steady-state percutaneous absorption of Azone is established after this initial change. Thus, Azone can enhance its own absorption as well as that of other compounds (Figure 7.4) (Wester et al., 1993b).

7.6 INDIVIDUAL VARIATION: *IN VITRO* HUMAN SKIN

It is well understood that chemical trials are designed with multiple volunteers to account for individual subject variation.

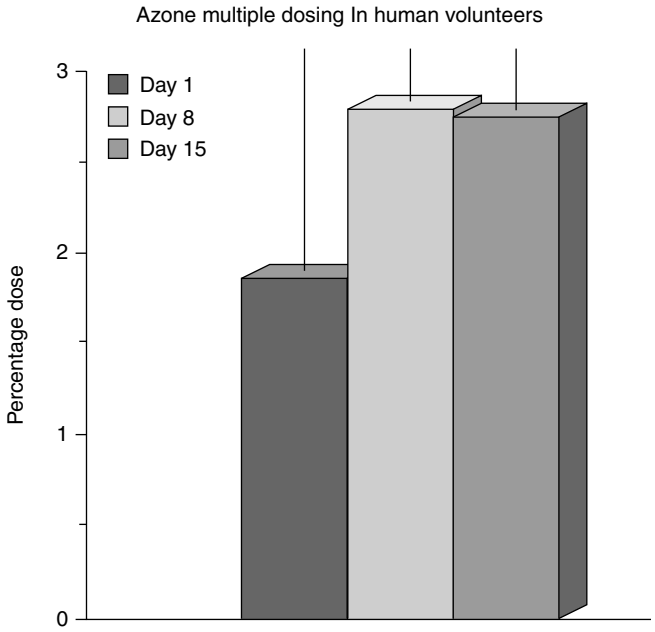


FIGURE 7.4 Azone *in vivo* in humans is able to enhance its own percutaneous absorption (day 1–8) until steady-state absorption is reached (day 1–15).

TABLE 7.7
***In Vitro* Percutaneous Absorption of Vitamin E Acetate into and through Human Skin**

	Percentage Dose Absorbed		
	Receptor Fluid	**Skin Content**	**Surface Wash**
		Formula A	
Skin Source			
1	0.34	0.55	74.9
2	0.39	0.66	75.6
3	0.47	4.08	89.1
4	1.30	0.96	110.0
Mean ± SD	0.63 ± 0.45[a]	1.56 ± 1.69[b]	87.4 ± 16.4
		Formula B	
Skin Source			
1	0.24	0.38	–
2	0.40	0.64	107.1
3	0.41	4.80	98.1
4	2.09	1.16	106.2
Mean ± SD	0.78 ± 0.87[a]	1.74 ± 2.06[b]	103.8 ± 5.0

[a] $p = .53$ (nonsignificant; paired t-test).
[b] $p = .42$ (nonsignificant; paired t-test).

This extends to *in vivo* percutaneous absorption where individual subject variability has been demonstrated (Wester and Maibach, 1985). This subject variation also extends to *in vitro* human skin samples (Wester and Maibach, 1991). Table 7.7 shows the *in vitro* percutaneous absorption of vitamin E acetate through human skin. Percent doses absorbed for two formulations, A and B, are shown for 24-h receptor fluid accumulation and for skin content (skin digested and assayed at 24-h time point). Assay of skin surface soap

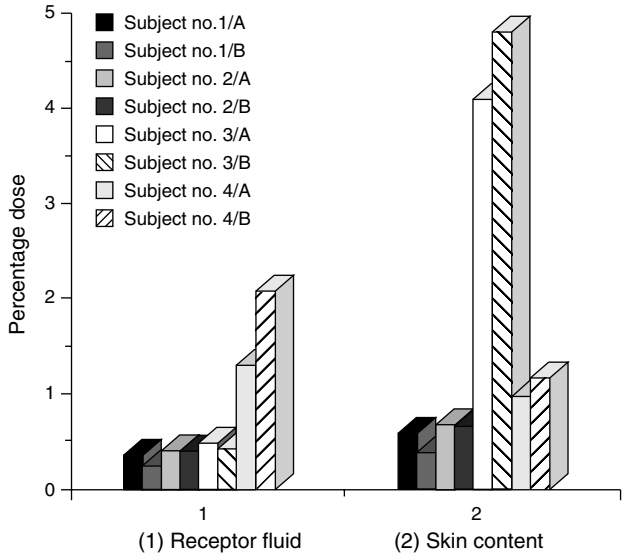


FIGURE 7.5 *In vitro* percutaneous absorption of vitamin E acetate in human skin. Note that individual variation is consistent between formulations A and B.

TABLE 7.8
Study Design for *In Vitro* Percutaneous Absorption

Human Skin Source	Treatments					
	A	B	C	D	E	F
1	X	X	X	X	X	X
2	X	X	X	X	X	X
3	X	X	X	X	X	X
4	X	X	X	X	X	X

Note: A–F can be separate treatment or replicates of treatments. If necessary or desired, human skin sources can be extended beyond 4.

and water washed at the end of the 24-h period gives dose accountability.

The two formulations were the same except for slight variation in pH. Statistically, there was no difference in absorption between the two formulations. However, a careful examination of the individual values in Table 7.7 shows consistency within individuals. Analysis of variance (ANOVA) for individual variation showed statistical significance for receptor fluid ($p = .02$) and skin content ($p = .000$) (Figure 7.5); therefore, when comparing treatments for *in vitro* percutaneous absorption, it is recommended that each treatment be a part of each skin source. Table 7.8 outlines a study based upon this.

7.7 MODELS: *IN VITRO* AND *IN VIVO*

Models are substitutes, and in the case of percutaneous absorption, the model substitutes for *in vivo* percutaneous absorption in humans. Models need to be validated, as shown for the rhesus monkey in Table 7.9. A popular substitute for humans *in vivo* is the use of human skin *in vitro*. Table 7.10

TABLE 7.9
***In Vivo* Percutaneous Absorption in Rhesus Monkey and Human (% Dose Absorbed)**

Compound	Rhesus Monkey	Human
2,4-Dinitrochlorobenzene	52 ± 4	54 ± 6
Nitrobenzene	4 ± 1	2 ± 1
Cortisone	5 ± 3	3 ± 2
Testosterone	18 ± 10	13 ± 3
Hydrocortisone	3 ± 1	2 ± 2
Benzoic acid	60 ± 8	43 ± 16
Diethyl maleate	68 ± 7	54 ± 7
DDT	19 ± 9	10 ± 4
Retinoic acid	2 ± 1	1 ± 0.2
2,4-D	9 ± 2	6 ± 2

Note: These are data collected over the years from many laboratories. The rhesus monkey is a good animal model to predict potential percutaneous absorption in humans.

gives the *in vitro* percutaneous absorption of several chemicals, expressed as percent dose accumulated in receptor fluid for 24 h, and the skin chemical content at that time frame. For DDT, benzo[a]pyrene, chlordane, pentachlorophenol, and polychlorinated biphenyls (PCBs), there is negligible receptor fluid accumulation (Wester et al., 1990, 1992a, 1993b, 1993d). A basic rule on *in vitro* percutaneous absorption is that solubility of a chemical in receptor fluid should not be the limiting step. Table 7.11 shows that these chemicals have high log *P* values. For example, a log *P* of 6.91 (DDT) means that when DDT is introduced into an equal amount of octanol and water, 6,910,000 molecules will end up in the octanol and one molecule will be in the water. The stratum corneum is lipophilic, so there is a tendency for chemicals to stay in skin and not partition into water-based receptor fluid. In Table 7.10, the skin content of chemical is better than that of receptor fluid, and somewhat predictive of *in vivo* absorption, but not in all cases (pentachlorophenol, PCBs) (Wester et al., 1990, 1992a, 1992b, 1993a, 1993b, 1993d).

7.8 PERCUTANEOUS ABSORPTION FROM CHEMICALS IN CLOTHING

Chemicals in cloth cause cutaneous effects. For example, Hatch and Maibach (1986) reported that chemicals added to cloth in 10 finish categories (dye, wrinkle resistance, water repellancy, soil release, and so on) caused irritant and allergic contact dermatitis, atopic dermatitis exacerbation, and urticarial and phototoxic skin responses. This is qualitative information that chemicals will transfer from cloth to skin *in vivo* in humans. Quantitative data are lacking. Snodgrass (1992) studied permethrin transfer from treated cloth to rabbit skin *in vivo*. Transfer was quantitative but less than expected. Interestingly, permethrin remained within the cloth after detergent laundering.

In other studies (Quan et al., 1994), *in vitro* percutaneous absorption of glyphosate and malathion through human skin

TABLE 7.10
***In Vitro* versus *In Vivo* Percutaneous Absorption**

		Percentage Dose		
			In Vitro	
Compound	**Vehicle**	**Skin**	**Receptor Fluid**	***In Vivo***
DDT	Acetone	18.1 ± 13.4	0.08 ± 0.02	18.9 ± 9.4
Benzo[a]pyrene	Acetone	23.7 ± 9.7	0.09 ± 0.06	51.0 ± 22.0
Chlordane	Acetone	10.8 ± 8.2	0.07 ± 0.06	6.0 ± 2.8
Pentachlorophenol	Acetone	3.7 ± 1.7	0.6 ± 0.09	29.2 ± 5.8
PCBs (1242)	Acetone	–[a]	–[a]	21.4 ± 8.5
	TCB	–[a]	–[a]	18.0 ± 3.8
	Mineral oil	10.0 ± 16.5	0.1 ± 0.07	20.8 ± 8.5
PCBs (1254)	Acetone	–[a]	–[a]	–[a]
	TCB	–[a]	–[a]	14.6 ± 3.6
	Mineral oil	6.4 ± 6.3	0.3 ± 0.6	20.8 ± 8.3
2,4-D	Acetone	–[a]	–[a]	8.6 ± 2.1
Arsenic	Water	1.0 ± 1.0	0.9 ± 1.1	2.0 ± 1.2
Cadmium	Water	6.7 ± 4.8	0.4 ± 0.2	–[a]
Mercury	Water	28.5 ± 6.3	0.07 ± 0.01	–[a]

[a] Study was not done.

TABLE 7.11
Octanol/Water Partition Coefficients of Compounds

Compound	**log *P***
DDT	6.91
Benzo[a]pyrene	5.97
Chlordane	5.58
Pentachlorophenol	5.12
2,4-D	2.81
PCBs mixture	4.80
Aroclor 1242	
Aroclor 1254	(high log P)

TABLE 7.12
***In Vitro* Percutaneous Absorption of Glyphosate and Malathion from Cloth through Human Skin**

Chemical	**Donor Conditions**	**Treatment**	**Percentage Dose Absorbed**
Glyphosate	1% Solution (water)	None	1.42 ± 0.25
	1% Solution on cloth	0 h	0.74 ± 0.26
	1% Solution on cloth	24 h	0.08 ± 0.01
	1% Solution on cloth	48 h	0.08 ± 0.01
	1% Solution on cloth	Add water	0.36 ± 0.07
Malathion	1% Solution (water/ethanol)	None	8.77 ± 1.43
	1% Solution on cloth	0 h	3.92 ± 0.49
	1% Solution on cloth	24 h	0.62 ± 0.11
	1% Solution on cloth	48 h	0.60 ± 0.14
	1% Solution on cloth	Add water/ethanol	7.34 ± 0.61

Note: Both glyphosate and malathion in solution (treatment = none) are absorbed through human skin. Glyphosate and malathion on cotton cloth show some absorption into skin, depending upon the time the chemical was added to cloth treatment = 0, 24, and 48 h). When the cloth was wetted (treatment = add water or add water/ethanol), the transfer of glyphosate and malathion from cloth to human akin was increased. This suggests that sweating, skin oil, or even rain may facilitate transfer of chemicals from cloth to skin.

was decreased when added to cloth (the cloth then placed on the skin) and this absorption decreased as time passed over 48 h (Table 7.12). It is assumed that with time the chemical will sequester into deep empty spaces of the fabric, (or some type of bonding is established between chemical and fabric. When water was added to glyphosate/cloth and water/ethanol to malathion/cloth, the percutaneous absorption increased malathion to levels from solution). This perhaps reflects clinical situations where dermatitis occurs most frequently in human sweating areas (axilla, crotch).

7.9 HUMAN *IN VIVO* PERCUTANEOUS ABSORPTION

7.9.1 Diazinon

Diazinon is an organophosphorus insecticide that, through general use, comes into contact with human skin. To investigate its percutaneous absorption, human volunteers were exposed for 24 h to ^{14}C-labeled diazinon applied in acetone solution (2 μg/cm^2) to the forearm or abdomen, or in lanolin wool grease (1.47 μg/cm^2) to the abdomen (Table 7.13).

TABLE 7.13
Percutaneous Absorption of Diazinon in Humans

Skin Site	Vehicle	Percutaneous Absorption (% of Dose)[a]
Forearm	Acetone	3.85 ± 2.16
Abdomen	Acetone	3.24 ± 1.94
Abdomen	Lanolin	2.87 ± 1.16

[a] Mean ± SD for six volunteers per group, calculated from human urinary ^{14}C disposition corrected for incomplete/other route excretion with the monkey urinary disposition after IV dosing.

Complete void urine samples were collected daily for 7 days. Percutaneous absorption ranged from 2.87 ± 1.16% (mean ± SD, $n = 6$) to 3.85 ± 2.16% of the applied amount, and there were no statistically significant differences with regard to site or vehicle of application. In rhesus monkeys, over the 7 days after IV dosing (2.1 μCi [^{14}C]diazinon, 31.8 μg), a total of 55.8 ± 68% ($n = 4$) of the dose was excreted in the urine, and 22.6 ± 5.2% was eliminated in the feces (78.4% total accountability). In *in vitro* percutaneous absorption studies with human abdominal skin, 14.1 ± 9.2% of the applied dose accumulated in the receptor fluid over 24 h of exposure to 0.25 μg/cm^2 (acetone vehicle). The calculated mass absorbed was the same (0.035 μg/cm^2) for both *in vitro* and *in vivo* absorption through human skin (Wester et al., 1993c).

7.9.2 Pyrethrin and Piperonyl Butoxide

To determine the human *in vivo* percutaneous absorption, a commercial formulation containing either [^{14}C]pyrethrin (3.8 mCi/mmol) or [^{14}C]piperonyl butoxide (3.4 mCi/mmol) was applied to the ventral forearm of six human volunteers (Table 7.14). The formulation contained 0.3% pyrethrin or 3.0% piperonyl butoxide. Spreadability studies showed that concentrations of 5.5 μg pyrethrin/cm^2 and 75.8 μg piperonyl butoxide/cm^2 (used in this study) would be consistent with levels found in actual use. The forearms were thoroughly cleansed with soap and water 30 min after application (as recommended for actual use). Percutaneous absorption was determined by urinary cumulative excretion following dose application. With a 7-day urinary accumulation, 1.9 ± 1.2% (SD) of the dose of pyrethrin and 2.1 ± 0.6% of the piperonyl butoxide applied was absorbed through the forearm skin. One hour after application, blood samples contained no detectable radioactivity. The percutaneous absorption of pyrethrin and piperonyl butoxide from the scalp was calculated to be 7.5% of the applied dose for pyrethrin and 8.3% for piperonyl butoxide. The calculated half-life of ^{14}C excretion was 50 h for pyrethrin and 32 h for piperonyl butoxide. The data should be of relevance to risk assessment where extrapolating animal data to humans (Wester et al., 1994b).

TABLE 7.14
Percutaneous Absorption of Pyrethrin and Piperonyl Butoxide from Human Forearm and Calculated from Human Scalp

	Dose Absorbed (%)			
	Pyrethrin		Piperonyl Butoxide	
Subject	Forearm	Scalp[a]	Forearm	Scalp[a]
1	1.4	5.6	2.8	1.2
2	1.6	6.4	1.8	7.2
3	2.0	8.0	2.8	11.2
4	0.6	2.4	1.8	7.2
5	1.6	6.4	1.4	5.6
6	4.1	16.4	1.8	7.2
Mean ± SD	1.9 ± 1.2	7.5 ± 4.7	2.1 ± 0.6	8.3 ± 2.4

[a] Scalp is assumed to have fourfold absorption greater than forearm.

TABLE 7.15
***In Vivo* Percutaneous Absorption of Isofenphos in Humans**

	Percentage Dose Absorbed[a]	
Subject	24-h Exposure	72-h Exposure
1	1.18	4.12
2	1.93	3.86
3	2.47	3.01
4	8.94	3.56
Mean ± SD	3.63 ± 3.58	3.64 ± 0.48

[a] Percentage dose absorbed = (urinary ^{14}C excretion for topical/urinary ^{14}C excretion for IV) × 100.

7.9.3 Isofenphos

Studies were done to determine the percutaneous absorption of isofenphos in human volunteers from whom informed consent had been obtained. *In vivo* absorption in humans was 3.6 ± 3.6% of applied dose for 24-h exposure and 3.6 ± 0.5% for 72-h exposure (Table 7.15). Skin wash recovery data showed that isofenphos evaporates from *in vivo* skin during the absorption process; the surface dose is minimal (<1%) by 24 h. Skin stripping showed no residual isofenphos in stratum corneum. This explains the similar absorption for 24- and 72-h prewash exposures. Skin surface recovery *in vivo* with soap and water was 61.4 ± 10.4% for the first dosing time (15 min). Time-recovery response declined with time to 0.5 ± 0.2% at 24 h. *In vitro* absorption utilizing flow-through diffusion methodology with human cadaver skin and human plasma receptor fluid gave 2.5 ± 2.0% dose absorbed, an amount similar to *in vivo* studies (Table 7.16). An additional 6.5 ± 24% was recovered in the skin samples (total of 9%). Skin surface wash at 24 h recovered 79.7 ± 2.2% and

TABLE 7.16
***In Vitro* Percutaneous Absorption of Isofenphos with Human Skin**

	Average Percentage Applied Dose[a]		
Human Plasma Receptor Fluid	**Skin**	**Surface Wash**	**Total**
2.5 ± 2.0	6.5 ± 2.4	79.7 ± 2.2	88.7 ± 4.6

[a] Mean ± SD for four replicates.

skin content was 6.5 ± 2.4% (total dose accountability of 88.7 ± 4.6%). Thus, isofenphos was available for absorption during the entire dosing period. Neither *in vitro* absorption nor *in vitro* evaporation studies predicted the potential skin evaporation of isofenphos. Published dermal studies in the rat had predicted isofenphos absorption at 47% of applied dose (12-fold greater than actual in humans). Subsequent toxicokinetic modeling predicted possible concern with the use of isofenphos. This is an example where the choice of the rat produced a nonrelevant absorption prediction. *In vivo* studies in human volunteers seem more relevant for predicting percutaneous absorption in humans (Wester et al., 1992c).

7.10 DISCUSSION

This chapter provides examples and discussion of information relating to relevance and validation of percutaneous absorption for risk assessment. The assessment of short-term exposure may be missed with some conventional calculations of percutaneous absorption such as conventional flux value (or modeled flux value) and lag time. *In vivo*, a chemical can rapidly partition into skin during a bath or swim (time period on the order of an hour or less). Interpretation of an artificial (due to *in vitro* diffusion system) lag time may eliminate that rapid uptake. Alternatively, a conventional flux rate, where linearity over time is scaled back to 30 min of exposure, may underestimate the actual exposure.

The other points raised in this chapter concern multiple exposures and absorption from nonformulated media (e.g., cloth/fabric). The question to be asked is, does the study design used to produce the (percutaneous absorption) data reflect the problem/risk assessment that is being investigated? A firmer understanding of these specific issues is needed, as well as more substantive methods of designing and interpreting clinically relevant situations.

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8 Percutaneous Absorption: 7 Roles of Lipids

Philip W. Wertz

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8.1 IMPLICATION OF STRATUM CORNEUM LIPIDS IN PERMEABILITY BARRIER FUNCTION

Experiments performed in the early 1950s in which the effects of organic solvents on permeability of the skin were studied implicated stratum corneum lipids in the function of the permeability barrier (Berenson and Burch, 1951; Blank, 1952). However, little further progress was made until 1973, when two breakthroughs were reported. Through the use of electron-dense water-soluble tracers, Squier (1973) was able to demonstrate by transmission electron microscopy that the permeability barrier starts at the bottom of the stratum corneum, where the contents of the lamellar granules are extruded. These studies also indicated that all layers of stratum corneum contribute to barrier function. In this same year, Breathnach et al. (1973) demonstrated by freeze fracture electron microscopy that the intercellular spaces of the stratum corneum contain multiple stacked, broad lipid sheets. These findings were further elaborated by Elias and Friend (1975). In the scenario that emerged from this body of work, lipids accumulate in lamellar granules, and are extruded into the intercellular spaces in the upper granular layer. After extrusion, the short stacks of lipid lamellae are transformed into multiple broad lipid sheets, which fill most of the intercellular spaces throughout the stratum corneum. It is this lamellar lipid in the intercellular spaces that determines the permeability of the stratum corneum.

8.2 ALTERATION OF LIPIDS WITH DIFFERENTIATION

Basal keratinocytes, like most mammalian cells, contain phospholipids and cholesterol as the principal lipids; however, as epidermal keratinocytes undergo differentiation there are dramatic alterations of lipid composition (Yardley and Summerly, 1981). In addition to more phospholipids, the differentiating cells synthesize a great deal of ceramides, glucosylceramides, and cholesterol. In the final stages of the differentiation program, the phospholipids are broken down and the glycolipids are deglycosylated, leaving ceramides, cholesterol, and fatty acids as the principal lipids of the stratum corneum (Wertz and Downing, 1989a). These are the lipids that form the intercellular lamellae. It is remarkable that unlike most biological membranes, the intercellular lamellae do not contain phospholipids.

8.3 LAMELLAR GRANULES

Much of the lipid that accumulates during epidermal differentiation is packaged into lamellar granules (Landmann, 1988). This small organelle is round to ovoid in shape, approximately 0.2 μm in diameter, and consists of one or several stacks of lamellar disks surrounded by a bounding membrane. The lamellar disks are thought to be flattened lipid vesicles.

Several investigators have examined the lipid composition of isolated lamellar granules, and have found the major lipid classes to be phospholipids, cholesterol, and glucosylceramides (Wertz et al., 1984; Freinkel and Traczyk, 1985; Grayson et al., 1985). One unusual glycolipid associated with lamellar granules consists of 30- through 34-carbon ω-hydroxyacids amide linked to sphingosine with glucose β-glycosidically attached to the primary hydroxyl group of the long-chain base and linoleic acid ester-linked to the ω-hydroxyl group (Wertz and Downing, 1983a;

Abraham et al., 1985). The ω-hydroxyacyl portion of this molecule is of sufficient length to span a typical lipid bilayer, and it has been proposed that the linoleate could insert into an adjacent bilayer, thus riveting the two together (Wertz and Downing, 1982). This sort of interaction could promote the flattening and stacking of lipid vesicles to produce the stacks of disks found within the lamellar granules. It has also been suggested that only about one-third of the acylglucosylceramide is associated with the internal lamellae, while the other two-thirds is in the bounding membrane (Wertz, 1992, 1996).

8.4 LIPID ENVELOPE

When the bounding membrane of the lamellar granule fuses into the cell plasma membrane, much lipid including acylglucosylceramide is introduced to the cell periphery. At about this time, a thick band of protein is deposited on the inner aspect of the plasma membrane (Rice and Green, 1977, 1979; Mehrel et al., 1990), and this protein becomes cross-linked via isopeptide (Abernathy et al., 1977; Rice and Green, 1977) and disulfide linkages (Polakowska and Goldsmith, 1991). The glucose is removed from the acylglucosylceramide through the action of a glucocerebrosidase (Wertz and Downing, 1989b). The linoleate is removed and possibly recycled (Madison et al., 1989), and the remaining ω-hydroxyceramide becomes attached through ester linkages to the outer surface of the cross-linked protein (Wertz and Downing, 1987). The amount of this covalently bound lipid is just sufficient to provide a monomolecular coating over the entire surface of the cornified cell (Wertz and Downing, 1987; Swartzendruber et al., 1987). In the porcine model, there is one ω-hydroxyceramide that contains sphingosine as the base (Wertz and Downing, 1987); however, in human stratum corneum, there are two additional ω-hydroxyceramides. One of these contains 6-hydroxyceramide as the base component (Wertz et al., 1989; Robson et al., 1994), and the other contains phytosphingosine (Hill et al., 2006). It appears that transglutaminase 1 may be responsible for attachment of the ω-hydroxyceramide molecules to involucrin in the envelope (Nemes et al., 1999).

8.5 CHEMICAL STRUCTURES OF THE STRATUM CORNEUM LIPIDS

The first completely described epidermal ceramides were those from porcine epidermis (Wertz and Downing, 1983b). These ceramides comprised 50% of the mass of the stratum corneum lipids (Wertz and Downing, 1989a) and were fractionated into six chromatographically distinct fractions by thin-layer chromatography. Each fraction was analyzed by a combination of chemical, chromatographic, and spectroscopic methods, and one unique structural type of ceramide was found in each fraction. Accordingly, these ceramides were called ceramides 1 through 6, in order of increasing polarity. The least polar of the porcine ceramides, ceramide 1, is derived from the above-mentioned acylglucosylceramide, and may serve as a molecular rivet in stabilizing the multilamellar sheets within the intercellular spaces. Ceramide 2 consists of mainly 24- through 28-carbon fatty acids amide-linked to sphingosines. Ceramide 3 contains the same range of fatty acids, but they are here amide-linked to phytosphingosines. Ceramides 4 and 5 both contain α-hydroxyacids amide-linked to sphingosines. They differ in that ceramide 4 contains 24- through 28-carbon hydroxyacids, whereas ceramide 5 contains mainly the 16-carbon α-hydroxypalmitic acid. Ceramide 6 consists of α-hydroxyacids amide-linked to phytosphingosines. Cholesterol, a ubiquitous membrane lipid, comprises 25% of the stratum corneum lipid mass (Yardley and Summerly, 1981; Wertz and Downing, 1989a). The free fatty acids comprise 10% of the stratum corneum lipid (Yardley and Summerly, 1981; Wertz and Downing, 1989a), and like those from ceramides 2 and 3, are mainly 24- through 28-carbon saturates. It is noteworthy that with the exception of the linoleate tail of ceramide 1 for which a special function has been proposed, all of the aliphatic chains in the stratum corneum lipids are straight with no methyl branches or *cis* double bonds, and the polar head groups are minimal. These structural features should favor formation of highly ordered, probably gel phase membrane domains, and this expectation is supported by infrared (Potts and Francoeur, 1993), x-ray, and thermal studies (Bowstra et al., 1991).

As noted, the ceramide structures described in the previous paragraph were from porcine epidermis (Wertz and Downing, 1983b); however, all of the same ceramides are present in human epidermis (Wertz et al., 1985, 1987). The structures of the human stratum corneum lipids are presented in Figure 8.1. These lipids are shown in order of increasing polarity from top to bottom. One exception to this order occurs with the sixth and seventh listed ceramides, which comigrate. They have been separated only after being isolated as a mixed fraction, being acetylated, and being rerun in a different chromatographic system (Ponec et al., 2003). In addition to long-chain, saturated fatty acids, cholesterol, and the ceramides found in porcine stratum corneum, human stratum corneum contains four additional types of ceramides. Three of these contain 6-hydroxysphingosine as the base component (Robson et al., 1994; Stewart and Downing, 1999), and one is an acylceramide analogous to porcine ceramide 1 but with phytosphingosine as the base component (Ponec et al., 2003).

Because of the additional ceramides in human stratum corneum and the fact that they do not all separate into discrete chromatographic fractions, the nomenclature system based on chromatographic fraction numbers is no longer adequate. A system of ceramide nomenclature proposed by Motta et al. (1993) is increasingly coming into use. Within this system the amide-linked fatty acid is designated by N, A, or O, for normal fatty acids, α-hydroxyacids, and ω-hydroxyacids, respectively. Similarly, the base component

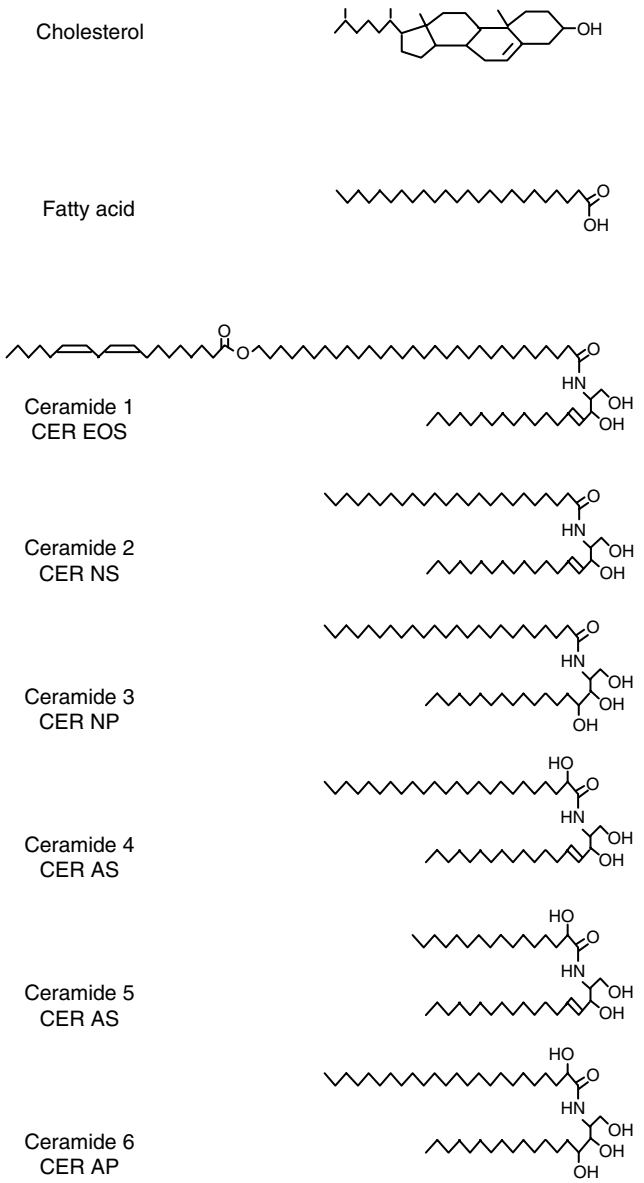


FIGURE 8.1 Representative structures of the major lipids from human stratum corneum. (From 6th edition.)

is designated as S, P, or H for sphingosine, phytosphingosine, and 6-hydroxysphingosine, respectively. If an ester-linked fatty acid is also present this is designated with a prefix E. The Motta nomenclature for each human ceramide is indicated in Figure 8.1.

8.6 ULTRASTRUCTURE OF THE INTERCELLULAR SPACES OF THE STRATUM CORNEUM

Although the intercellular lamellae of the stratum corneum could be detected by the freeze fracture method (Breathnach et al., 1973), this technique provides relatively little information. Until the recent introduction of ruthenium tetroxide as a postfixative for the preparation of samples (Madison et al., 1987), the intercellular lamellae could not be visualized by transmission electron microscopy, and it was proposed that the lipids were removed during sample processing (Elias et al., 1988). However, it is now known that this is not the case. The stratum corneum lipids, as appropriate for materials that interface with the external environment, are relatively chemically inert, and do not react appreciably with osmium tetroxide used for routine fixation. This difficulty was overcome by substitution of the stronger oxidizing agent, ruthenium tetroxide. With this reagent the intercellular lamellae can be routinely visualized as shown in Figure 8.2. In the nine-band pattern shown in the figure, the first lucent band adjacent to the electron-dense protein band of the cornified envelope is the covalently attached lipid. The paired bilayers are produced by edge-to-edge fusion of flattened lipid vesicles extruded from the lamellar granules (Landmann, 1988). The narrow lucent bands in the center of the nine-band pattern has been thought to be formed by eversion of linoleate chains from acylceramide molecules in the adjacent lamellae to form an interdigitated zipper-like central narrow lamella (Swartzendruber et al., 1989; Wertz, 1996). In this interpretation, space vacated by everted chains and remaining within the framework are filled in by free lipids. Essentially, the same model for the broad–narrow–broad repeat units has been arrived at independently in an attempt to explain the 13 nm repeat seen in x-ray diffraction studies (Bouwstra et al., 2000, 2002). This has been called the sandwich model and features trilaminar units with outer gel phase lamellae and a central narrow fluid lamella (Bouwstra et al., 2000, 2001).

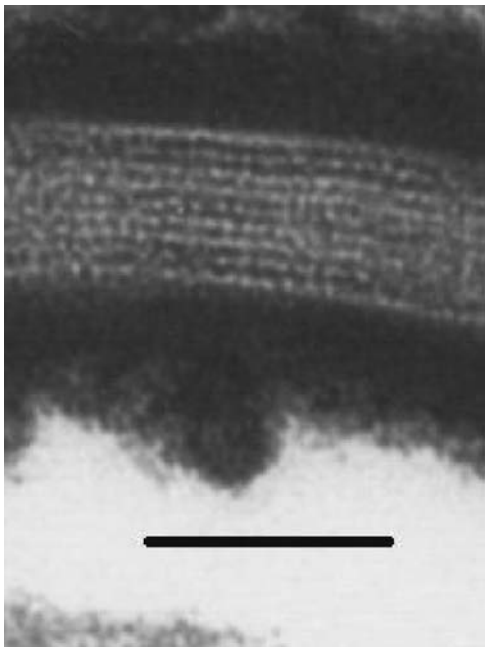


FIGURE 8.2 Transmission electron micrograph of stratum corneum after fixation with ruthenium tetroxide. Bar = 40 nm. (From 6th edition.)

In both of these broad–narrow–broad models of the intercellular lamellae, linoleate tails from the acylceramide molecules are thought to insert into the central narrow lamella with interdigitation. The remaining portion of the acylceramide molecules are thought to be in the outer lamellae. Thus, the acylceramides link the three lamellae together and fluidize the central lamella. In both models, the overall dimension of the trilaminar units is 13 nm. Recently, these models have been challenged (Hill and Wertz, 2003). Basically, it has been proposed that all three lamellae within the 13 nm trilaminar units are approximately 4.3 nm in thickness with acylceramide linoleates inserted into the central lamella. It was pointed out that this arrangement would result in relatively saturated outer lamellae and a concentration of double bonds from linoleate in the central lamellae. These double bonds would make the central lamella capable of reducing more ruthenium compared to the outer lamellae. This additional reduced ruthenium would accumulate beneath the planes of the polar headgroups of the central lamella, thus creating the false appearance of broad–narrow–broad lamellae. Strong support for this model comes from the application of cryo-transmission electron microscopy to the stratum corneum. This method avoids the artifacts associated with the use of chemical fixatives. It demonstrates intercellular lamellae in the stratum corneum that are uniform in thickness and are approximately 4.3 nm thick (Norlen, 2003). The nine-band pattern shown in Figure 8.2 and a similar six-band pattern are the most common arrangements seen between adjacent layers of cells in human stratum corneum using the ruthenium tetroxide method. Results obtained using cryo-transmission electron microscopy of vitreous, fully hydrated human epidermis suggest that the number of lamellae across the intercellular spaces may actually be greater (Norlen, 2003).

A single trilaminar unit with the typical broad–narrow–broad appearance is frequently seen between the ends of adjacent corneocytes. It has been suggested that the outer lamellae are the covalently bound hydroxyceramides and that the central lamella contains the sphingosine chains from these hydroxyceramides. It is proposed that the *trans* double bonds and additional hydroxyl groups from the long-chain bases again result in greater reduction of ruthenium within the central lamella resulting in a false impression of broad–narrow–broad lamellae (Hill et al., 2003).

Although the three-, six-, and nine-band patterns are the most frequent lamellar arrangements seen in normal epidermal stratum corneum when using the ruthenium tetroxide technique, the number and arrangement of lamellae is highly variable, and caution should be taken in comparing intercellular material in normal versus diseased or experimentally manipulated stratum corneum using this technique. The emergence and development of the cryo-transmission electron microscopic technique provides an alternative that appears less subject to artifacts.

It should be pointed out that the 13 nm trilaminar units can be reconstituted *in vitro* from either natural stratum corneum lipids (Kuemper et al. 1998) or from lipids of cholesterol, fatty acids, and suitable synthetic ceramides (de Jager et al., 2005). These reconstituted lamellae could be used for studies of physical properties, and given the availability of synthetic ceramides, a standardized model system could be established for *in vitro* studies on transdermal drug delivery.

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9 Chemical Partitioning into Powdered Human Stratum Corneum: A Useful *In Vitro* Model for Studying Interactions of Chemicals and Human Skin

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9.1 INTRODUCTION

Chemical delivery/absorption into and through the skin is important in both dermato-pharmacology and dermato-toxicology. The human stratum corneum (SC) is the first layer of the skin, and constitutes a rate-limiting barrier to the transport of most chemicals across the skin (Blank, 1965). Chemicals must first partition into the SC before entering the deeper layers of the skin, the epidermis and the dermis, to reach the vascular system. Chemical partitioning proceeds much faster than complete diffusion through the whole SC, and the process quickly reaches equilibrium (Scheuplein and Bronaugh, 1983). In addition to binding within the SC, a chemical can also be retained within the SC as a reservoir (Zatz, 1993). Thus, understanding the process of chemical partitioning into the SC becomes important in developing an insight into its barrier properties and transport mechanisms.

Human SC has been used for decades as an *in vitro* model to explore both percutaneous absorption and the risks associated with dermal exposure (Surber et al., 1990; Potts and Guy, 1992). The human SC includes the horny pads of palms and soles (callus), and the membranous SC covers the remainder of the body (Barry, 1983). The traditional method of preparation is via physical–chemical and enzymological processes to separate the membranous layers of the SC from whole skin (Juhlin and Shelly, 1977; Knufson et al., 1985). However, it is time consuming and, in some cases, it is difficult to control the size and thickness of a sheet of SC. Moreover, it is often difficult to locate a suitable skin source.

Powdered human stratum corneum (PHSC) prepared from callus (sole) is thus substituted for the intact membranous SC. Podiatrists routinely remove and discard PHSC from the human foot, so it is easily obtained. The callus can be cut easily and quickly into smaller pieces, and ground with dry ice to form a powder. In our laboratory, PHSC particle sizes between 180 and 300 μm were selected with the aid of a suitable sieve. Because a corneocyte is only about 0.5 μm thick and about 30–40 μm long, the selected PHSC contains both intact corneocytes and intercellular medium structures, and thus retains its original physical–biochemical properties. Moreover, the greater surface area of the PHSC enhances solute penetration. In a typical experimental procedure, a test chemical in a transport vehicle—water—is mixed with the PHSC, and the mixture is incubated. After a predetermined time period, a solution is separated from the PHSC by centrifugation, and samples are measured (Hui et al., 2000).

This chapter reviews PHSC as an *in vitro* model for studying chemical interaction with human skin, with reference to studies conducted in our laboratory over the last decades. The results demonstrate that PHSC (callus) offers an experimentally easy *in vitro* model for the determination of chemical partitioning into the SC and may be useful in many skin research areas.

9.2 PHSC AND PHYSICAL–CHEMICAL PROPERTIES OF STRATUM CORNEUM

The callus is derived from human SC, and thus should retain some of its physical and chemical characteristics (Wester et al., 1987). SC lipid plays an important role in the determination of skin functions. However, the average lipid content of the SC varies regionally, from 2.0, 4.3, 6.5, to 7.2 weight percent of dry SC from plantar, leg, abdomen, and face, respectively (Lampe et al., 1983). Table 9.1 shows that the average lipid content of the dry PHSC samples derived from various regions was 2.29 ± 0.25 weight percent after extraction. This result is consistent with that in human plantar as determined by Lampe et al. (1983).

The water content of the SC is of importance in maintaining SC flexibility. Three possible mechanisms of water absorption or retention capacity of the SC have been suggested: (i) Imokawa et al. (1986) suggested that SC lipids play a critical role because their removal by the application of acetone/ether decreased absorption/retention capacity. (ii) Friberg et al. (1992), however, considered that protein might also play an important role in SC water retention. They found that the additional water absorbed after reaggregation of equilibrated lipids and proteins was equally partitioned between the protein and the natural lipid fraction of the human SC. (iii) Middleton (1968) considered that water-soluble substances were responsible for water retention and for most of the extensibility of the corneum. He found that powdered SC—but not the intact corneum extracted by water—exhibited lower water-retention capacity. He suggested that the powdering procedure ruptures the walls of the corneum cells and allows water to extract the water-soluble substances without a prior solvent extraction. We measured the water-retention capacities of untreated PHSC, delipidized PHSC (as the protein fraction), and the lipid content, by measuring the amount of [3H]-water (μg equivalent) per mg PHSC after equilibration. As shown in Table 9.1, no statistical differences ($p > 0.05$) were observed for untreated PHSC, delipidized PHSC, and the combination of delipidized PHSC and the lipid content. The PHSC can absorb up to 49% by weight of dry untreated PHSC (Table 9.1), which is consistent with literature reports. Middleton (1968) found that the amount of water bound to intact, small pieces, and powdered guinea-pig footpad SC was 40, 40, and 43% of dry corneum weight. Leveque and Rasseneur (1988) demonstrated that the human SC was able to absorb water up to 50% of its dry weight. Our results (Table 9.1) suggest that the protein domain of the PHSC plays an important role in the absorption of water. Depletion of the PHSC lipid content did not affect water retention (Hui et al., 1993).

9.3 PHSC AND CHEMICAL PARTITIONING

Table 9.2 shows the effect of varying initial chemical concentrations on the PC PHSC/w of these compounds (Hui et al., 1993). Under fixed experimental conditions—2 h incubation time and 35°C incubation temperature—the concentration required to attain a peak value of the partition coefficient varied from chemical to chemical. After reaching the maximum, increases in the chemical concentration in the vehicle did not increase the PC value; rather, it slightly decreased or was maintained at approximately the same level. This is consistent with the results of Surber et al. (1990a,b) on whole SC. Chemical partitioning from the vehicle into the SC involves processes in which molecular binding occurs at certain sites of the SC, as well as simple partitioning. Equilibration of partitioning is largely dependent on the saturation of the chemical binding sites of the SC

TABLE 9.1
Lipid Content and Water Uptake of Powdered Human Stratum Corneum

Stratum Corneum Source	Lipid Content (% w/w Dry PHSC)	Water Uptake (μg/mg Dry PHSC)			
		Untreated PHSC	Delipidized PHSC		
			Lipid[a]	Protein[b]	Total
1	2.38	495.85	26.44	452.40	478.84
2	2.21	452.49	39.26	364.96	404.22
3	2.39	585.62	23.09	498.40	521.49
4	2.69	554.27	40.05	492.31	532.36
5	2.08	490.04	49.86	363.30	413.16
6	2.01	381.61	14.82	324.18	339.00
Mean	2.29	493.31	32.26	415.92	448.18
SD	0.25	72.66	12.97	74.50	75.47

[a] Lipid part extracted from the PHSC.

[b] Rest part of the PHSC after lipid extraction.

TABLE 9.2
Effect of Initial Aqueous Phase Chemical Concentration on Powdered Human Stratum Corneum/Water Partition Coefficient

Chemical[a] Log PC (o/w)	Concentration (%, w/v)	Partition (Mean)	Coefficient[b] (SD)
Dopamine	0.23	5.42	0.22
(−3.40)	0.46	6.04	0.28
0.92	5.74	0.28	
Glycine	0.05	0.36	0.01
(−3.20)	0.10	0.40	0.02
Urea	0.03	0.26	0.02
(−2.11)	0.06	0.15	0.02
	0.12	0.17	0.02
Glyphosate	0.02	0.79	0.04
(−1.70)	0.04	0.68	0.04
	0.08	0.70	0.01
Theophylline	0.18	0.37	0.02
(−0.76)	0.36	0.43	0.03
	0.54	0.42	0.02
Aminopyrine	0.07	0.44	0.09
(0.84)	0.14	0.46	0.03
Hydrocortisone	0.09	0.37	0.01
(1.61)	0.18	0.34	0.01
	0.36	0.29	0.02
Malathion	0.47	0.50	0.09
(2.36)	0.94	0.40	0.03
	1.88	0.53	0.04
Atrazine	0.09	0.53	0.06
(2.75)	0.14	0.59	0.07
	0.19	0.58	0.03
2,4-D	0.27	7.52	0.81
(2.81)	0.54	7.53	1.01
	0.82	8.39	1.67
Alachlor	0.32	1.11	0.05
(3.52)	0.64	1.08	0.04
	1.28	1.96	0.15
PCB	0.04	1237.61	145.52
(6.40)	0.08	1325.44	167.03
	0.16	1442.72	181.40

[a] Log PC (o/w) was cited in Hansch and Leo (1979).
[b] PC PHSC/water represent the mean of each test ($n = 5$) ± SD (Hui et al., 1993).

(Surber et al., 1990a; Rieger, 1993). The results also indicate that, under given experimental conditions, the maximum degree of partitioning was compound specific. As the SC contains protein, lipids, and various lower molecular weight (MW) substances with widely differing properties, the many available binding sites display different selective affinities with each chemical. Thus, the degree of maximum binding or of equilibration varies naturally with molecular structure (Rieger, 1993). This result demonstrated that the solubility limit of a compound in the SC was important in determining the degree of partitioning, as suggested by Potts and Guy (1993). This view has been further supported by our recent study. PHSC incubated with six chemicals in saturated solution at three different pH ranges (1 ~ 3, 6 ~ 8, and 10 ~ 14), and percent-binding rate was then counted. Table 9.3 shows the result that the percent-binding rate of PHSC is correlated with the solubility of the test chemicals regardless of their MWs or p*K*a values. Moreover, an interesting finding is that when the pH value is lower (2 ~ 3), it seems to favor the binding rate for the chemicals with positive log *P*, whereas the higher pH value (9 ~ 13) favors those chemicals with negative log *P*. For chemicals with log *P* values near neutral, pH values do not affect the PHSC binding rate. On the basis of the solubility limit of a chemical, the absorption process of water-soluble or lipid-soluble substances was controlled by the protein domain or the lipid domain, respectively or a combination of two (Raykar et al., 1988). Since the lipophilicity of the lipid domain in the SC is much higher than that of water, a lipophilic compound would partition into the SC in preference to water. Thus, when water is employed as the vehicle, the PC PHSC/w increases with increasing lipophilicity of solute (Scheuplein and Bronaugh, 1983). Conversely, the protein domain of the SC is significantly more polar than octanol and governs the absorption of hydrophilic chemicals (Rayker et al., 1988). For very lipophilic compounds, low solubility in water rather than increased solubility in the SC can be an important factor (Scheuplein and Bronaugh, 1983). Moreover, in addition to partitioning into these two domains, some amount of chemicals may be taken into the SC as the result of water hydration. This is the "sponge domain," named by Raykar et al. (1988). They assume that this water, having the properties of bulk water, carries an amount of solute into the SC equal to the amount of solute in the same volume of bathing solution. Therefore, for hydrophilic compounds and some lower lipophilic compounds, the partitioning process may include both the protein domain and sponge domain.

9.4 PHSC AND PERCUTANEOUS ABSORPTION

To evaluate sensitivity of this in the *in vitro* PHSC model, we examined chemical partitioning into the PHSC, as well as that *in vitro* percutaneous absorption in human skin, and *in vivo* percutaneous absorption in the rhesus monkey. Table 9.4 shows that the *in vivo* percutaneous absorption of nitroaniline from surface water following 30 min exposure was 4.1 ± 2.3% of the applied dose. This is comparable with the 5.2 ± 1.6% for *in vitro* absorption with human cadaver skin and the 2.5 ± 1.1% bound to PHSC. Wester et al. (1987) suggest that this methodology—the systems tested, binding to PHSC, and *in vitro* and *in vivo* absorption—can be used to predict the burden on the human body imposed by bathing or swimming.

9.5 PHSC AND THE SKIN BARRIER FUNCTION

The barrier function of the SC is attributed to its multilayered wall-like structure in which terminally differentiated keratin-rich epidermal cells (corneocytes) are embedded in an intercellular lipid-rich matrix. Any physical factor or chemical reagent that interacts with this two-compartment

TABLE 9.3
Effect of pH Change on Chemicals Binding Powdered Human Stratum Corneum in Saturated Concentration

	Parameters				Percentage Dose Bound[a]		
Chemicals	**MW**	**Solubility**	**Log *P***	**p*Ka***	**pH = 1 ~ 3**	**pH = 6 ~ 8**	**pH = 10 ~ 14**
Salicylic acid	138	2240	2.26	2.97	17.67 (1.38)	3.75 (0.21)	0.42 (0.12)
Hydroquinone	110	7200	0.59	10.9	2.45 (0.38)	1.33 (0.26)	0.71 (0.12)
Urea	60	545,000	−2.11	0.1	0.66 (0.37)	0.41 (0.39)	2.47 (1.86)
Glycerol	92	1,000,000	−1.76	14.4	0.08 (0.03)	0.08 (0.02)	0.35 (0.07)
Propylene glycol	76	1,000,000	−0.92	14.9	0.11 (0.04)	0.17 (0.07)	0.11 (0.01)
Caffeine	194	21,600	−0.07	10.4	11.69 (1.84)	13.03 (1.90)	10.47 (4.11)

[a] Each number represents the mean (SD) of six samples.

TABLE 9.4
***In Vivo* Percutaneous Absorption of *p*-Nitroaniline in the Rhesus Monkey Following 30 min Exposure to Surface Water: Comparisons to *In Vitro* Binding and Absorption**

Phenomenon	**Percentage Dose Absorbed/Bound[a]**
In vivo percutaneous absorption, rhesus monkey	4.1 ± 2.3
In vitro percutaneous absorption, human skin	5.2 ± 1.6
In vitro binding, powdered human SC	2.5 ± 1.1

[a] Each number represents the mean ± SD of four samples.

TABLE 9.5
Protein Releasing from Powdered Human Stratum Corneum Following Chemical/Water Exposure

	Protein Content (mg/4 mL)[a]		
Test Chemicals	**10 min**	**40 min**	**24 h**
Glycolic acid	0.093 (0.026)	0.175 (0.029)	0.173 (0.041)
Sodium hydroxide	0.419 (0.054)	0.739 (0.301)	5.148 (1.692)
Water	0.002 (0.014)	0.135 (0.043)	0.077 (0.021)

[a] Each number represents the mean (SD) of six samples.

structure can affect the skin barrier function. Barry (1983) described how certain compounds and mechanical trauma can easily dissociate callus cells and readily dissolve their membranes. Thus the amount of protein (keratin) released from the SC after chemical exposure may be a measure of the solvent potential of the chemical. To evaluate this hypothesis, a test chemical in water is mixed with PHSC and incubated. After a predetermined time period, a solution is separated from the PHSC by centrifugation. The protein (keratin) content of the solution is then measured. Table 9.5 shows the amount of protein released from the PHSC after incubation with glycolic acid, sodium hydroxide, or water alone, at different time points. Sodium hydroxide has a pronounced ability to release protein from PHSC. This ability increases with increasing incubation time. The results suggest that the PHSC model constitutes a vehicle to probe the barrier nature of the SC and the chemical interactions with the PHSC.

9.6 PHSC AND DISEASED SKIN

PHSC has potential application in medical treatment. For instance, a set of vehicles can be screened to determine which vehicle most readily releases a given drug into the SC. This information would assist in the determination of the most effective approaches to drug delivery via the skin. Furthermore, diseases involving the SC can be studied using PHSC. An example in Table 9.6 is the partitioning of hydrocortisone from normal and psoriatic PHSC. In this case, we have shown that there is no difference in partitioning between normal and psoriatic PHSC. It should be noted that there is no difference between *in vivo* percutaneous absorption of hydrocortisone in normal volunteers and that in psoriatic patients (Wester et al., 1983).

TABLE 9.6
Aqueous Partition Coefficient of Hydrocortisone with Normal and Psoriatic Stratum Corneum

	Partition Coefficient[a]	
Stratum Corneum Type	**Mean**	**S.E.**
Normal sheet (abdominal)	1.04	0.88
Normal powdered (plantar)	1.70	0.47
Psoriatic	1.94	0.42

[a] No statistical significance ($P > 0.05$).

9.7 PHSC AND ENVIRONMENTALLY HAZARDOUS CHEMICALS

The leaching of environmentally hazardous chemicals from soil and their absorption by the skin of a human body is a major concern. Knowledge of the extent and degree of such absorption will aid in determining the potential health hazards of polluted soil. Our laboratory's interest is in the potential percutaneous absorption of contaminants from soil. Soil can be readily mixed with PHSC, but centrifugation does not separate the two. However, centrifugation readily separates PHSC from any liquid, to varying degrees. Thus, the partition coefficients of various liquids may be determined relative to a common third liquid. These relative partitions can then be compared to those of other compounds and skin absorption values (Wester et al., 1992, 1993a,b) to evaluate the degree of hazard. We have determined such coefficients for several environmentally hazardous chemicals partitioning from soil into PHSC (Table 9.7).

9.8 PHSC AND CHEMICAL DECONTAMINATION

Our laboratory uses the PHSC model to determine which chemicals might be able to remove (decontaminate) hazardous chemicals from human skin. A contaminant chemical is mixed with PHSC, and the decontaminant effects of a series of possible decontaminants measured. The liquid decontaminant is mixed with contaminated PHSC and, after a predetermined time period, a solution is separated from the PHSC by centrifugation. The content of the solution is a measure of decontaminant's potential. This is shown in Table 9.8, which demonstrates that alachlor readily contaminates PHSC. Water alone removes only a small portion of the alachlor. However, a 10% soap solution removes a larger portion of the alachlor, and 50% soap solution removes most of it. Perhaps this is an elegant way to show that soapy water is effective in washing one's hands. However, it does illustrate the use of PHSC to determine the effectiveness of skin decontamination (Scheuplein and Blank, 1973).

TABLE 9.7
Partition Coefficients of Four Environmentally Hazardous Chemicals in PHSC/Water and Soil/Water

	Partition Coefficient	
Test Chemicals	**PHSC**	**Soil**
Arsenic	1.1×10^4	2.4×10^4
Cadmium chloride	3.6×10^1	1.0×10^5
Arodor 1242	2.6	1.7
Arodor 1254	2.9	2.0

TABLE 9.8
Decontaminants Selection to Remove Environmentally Hazardous Chemical (Alachlor) from Human Skin

	[14C]-Alachlor
	(% Dose)
PHSC	90.3 ± 1.2
Alachlor in Lasso supernatant	5.1 ± 1.2
Water-only wash of PHSC	4.6 ± 1.3
10% Soap and water wash	77.2 ± 5.7
50% Soap and water wash	90.0 ± 0.5

Note: [^{14}C]-Alachlor in Lasso EC formulation (1:20 dilution) mixed with powdered human SC, let set for 30 min, then centrifuged. SC wash with (1) water only, (2) 10% soap water, and (3) 50% soap and water.

9.9 PHSC AND ENHANCED TOPICAL FORMULATION

Macromolecules have attracted interest as potential drug entities, and as modulators to percutaneous delivery systems. Two macromolecular polymers (MW 2081 and 2565) were developed to hold cosmetics and drugs to the skin surface by altering the initial chemical and skin partitioning. The effect of these polymers on the partition coefficient of estradiol with PHSC and water was determined in our laboratory. As shown in Table 9.9, the polymer L had no effect on the estradiol PC between PHSC and water. The polymer H, however, showed a significant increase ($P < 0.01$) in log PC for estradiol concentrations of 2.8 and 0.25 mg/mL. This increase was dependent on the polymer concentration (Wester et al., 2002). The results suggest that the PHSC model can help in the development and selection of enhanced transdermal delivery systems.

9.10 PHSC AND QUANTITATIVE STRUCTURE–ACTIVITY RELATIONSHIPS PREDICTIVE MODELING

Many experiments have been conducted to predict chemical partitioning into the SC *in vitro*. However, most were based on quantitative structure–activity relationships (QSARs)

TABLE 9.9
Effect of Two Polymers (L and H) on the Estradiol PC between PHSC and Water

Test Formulation and Polymer Concentration	Log PC PHSC/Water (Mean ± SD, $n = 5$)		
	Estradiol Concentration (μg/mL)		
	2.8	**0.028**	**0.028**
Polymer H (hydrophilic polymer, %)			
10	2.31 ± 0.22[a]	2.36 ± 0.14[a]	2.13 ± 0.07[a]
5	1.93 ± 0.10[b]	2.06 ± 0.21[b]	1.94 ± 0.06[b]
1	1.71 ± 0.10	1.61 ± 0.19	1.59 ± 0.26
Polymer L (lipophilic polymer, %)			
10	1.74 ± 0.10	1.65 ± 0.07	1.61 ± 0.14
5	1.70 ± 0.20	1.62 ± 0.17	1.65 ± 0.09
1	1.59 ± 0.19	1.57 ± 0.15	1.71 ± 0.07
Control (no polymer)	1.62 ± 0.14	1.68 ± 0.11	1.71 ± 0.13

[a] Statistically significantly different from control ($P < 0.01$).
[b] Statistically significantly different from control ($P < 0.05$).

or related chemicals to determine the partitioning process, and few studies focus on structurally unrelated chemicals. Since the range of molecular structure and physicochemical properties is very broad, any predictive model must address a broad scope of partitioning behavior.

This study assesses the relationship of a number of chemicals with a broad scope of physicochemical properties in the partitioning mechanism between PHSC and water. Uniqueness and experimental accuracy are added by using PHSC. The experimental approach is designed to determine how the PC PHSC/w is affected by (1) chemical concentration, (2) incubation time, and (3) chemical lipophilicity (or hydrophilicity), and other factors. These parameters are used to develop an *in vitro* model that will aid in the prediction of chemical dermal exposure to hazardous chemicals.

Figure 9.1 describes a smooth, partially curvilinear relationship between the log PC PHSC/w and the log PC o/w of a number of chemicals. The lipophilicities and hydrophilicities of compounds were defined as log PC o/w larger or smaller than 0, respectively. For lipophilic chemicals such as aminopyrine, hydrocortisone, malathion, atrazine, 2,4-D, alachlor, and PCB, the logarithms of PHSC/w partition coefficients are proportional to the logarithms of the octanol/water partition coefficients.

$$\begin{aligned} &\log \text{PC PHSC/w} = 0.59 \log \text{ PC o/w} - 0.72 \\ &\text{Student } t \text{ values}: 9.93 \\ &n = 7 \quad r^2 = 0.95 \quad S = 0.26 \quad F = 98.61 \end{aligned} \tag{9.1}$$

For hydrophilic chemicals such as theophylline, glyphosate, urea, glycine, and dopamine, the log PC PHSC/w values are approximately and inversely proportional to log PC o/w.

$$\begin{aligned} &\log \text{PC PHSC/w} = -0.60 \log \text{PC o/w} - 0.27 \\ &\text{Student } T \text{ values}: -4.86 \\ &n = 5 \quad r^2 = 0.88 \quad s = 0.26 \quad F = 23.61 \end{aligned} \tag{9.2}$$

However, the overall relationship of the PC PHSC/w of these chemicals and their PC o/w is nonlinear. This nonlinear relationship is adequately described by the following equation:

$$\begin{aligned} &\log \text{PC PHSC/w} = 0.078 \log \text{PC o/w}^2 + 0.868 \\ &\log \text{MW} - 2.04 \\ &\text{Student } T \text{ values}: 8.29 \qquad 2.04 \\ &n = 12 \quad r^2 = 0.90 \quad s = 0.33 \quad F = 42.59 \end{aligned} \tag{9.3}$$

The logarithm of MW gave a stronger correlation in this regression than MW ($T = 1.55$) itself. In Figure 9.1, the calculated log PC PHSC/w (Y estimate) values are compared to the corresponding observed values for these chemicals. As shown, the calculated values are acceptably close to the observed values. The correspondence with minimal scatter suggests that this equation would be useful in predicting *in vitro* partitioning in the PHSC for important environmental chemicals (Hui et al., 1993).

9.11 DISCUSSION

A new *in vitro* model employing PHSC (callus) to investigate the interaction between chemicals and human skin has been developed in our laboratory. The PHSC (callus) offers

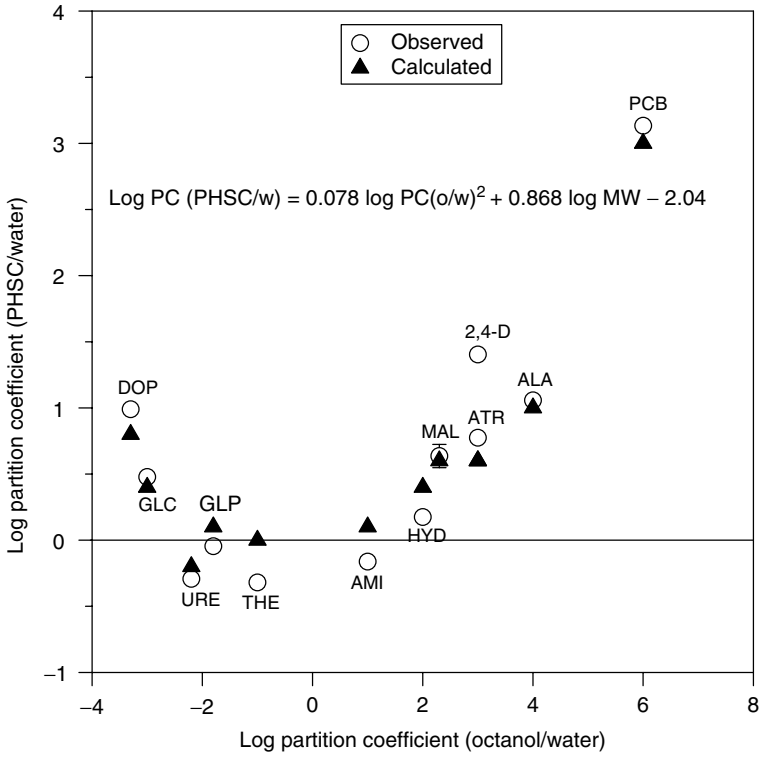


FIGURE 9.1 Correlation of the logarithm of SC/water partition coefficients (log PC sc/w) and logarithm of octanol/water partition coefficients of the 12 test chemicals. Open symbols are expressed as observed values and each represented the mean of a test chemical ± SD (n = 5). Close symbols are expressed as calculated values by the Equation 9.3. DOP = dopamin; GLC = glycine; URE = urea; GLP = glyphosate; THE = theophylline; AMI = aminopyrine; HYD = hydrocortisone; MAL = malathion; ATR = atrazine; 2,4-D = 2,4-dichlorophenoxyacetic acid; ALA = alachlor; PCB = polychlorinated biphenyls.

an experimentally easy *in vitro* model for the determination of chemical partitioning from water into the SC. Owing to the heterogeneous nature of the SC, the number and affinity of the SC binding sites may vary from chemical to chemical, depending upon molecular structure. For most lipophilic compounds, the PC PHSC/w were governed by the lipid domain, whereas PCs of the more hydrophilic compounds are determined by the protein domain and possibly, by the sponge domain (Raykar et al., 1988). These relationships can be expressed by the log PC PHSC/w of these chemicals as a function of the corresponding square of log PC o/w and log MW (Equation 9.3), which is useful in predicting various chemical partitionings into the SC *in vitro*. However, a disadvantage in using the human callus is that it may display some differences in water and chemical permeation when compared to membranous SC (Barry, 1983).

This chapter has summarized a variety of potential applications for PHSC, ranging from basic science to applications in medicine and environmental impact studies. PHSC, imagination, and a balanced study design can add to scientific knowledge.

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10 Sensitive Skin

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"Sensitive skin" is a term for several skin problems, which is widely used particularly by numerous skin patients, the media, and the drug and cosmetic companies. Today, hardly any cosmetic preparation without the label "for sensitive skin" can be found. Indeed, what is "sensitive skin?"

For many patients, sensitive skin stands in general for allergic reactions to common contact allergens, for example, nickel.[1,2] Their main problems are eczematous skin reactions by contact (e.g., in costume jewelry), or by ingestion in food, followed by hematogenic eczema.[3,4]

Other patients who are particularly affected by the problem of sensitive skin are atopic individuals or patients with a disrupted epidermal barrier function.[5–9] These individuals develop dermatitis caused by numerous triggers. Exogenous triggers (e.g., chemical or mechanical irritation, allergens, climatic conditions, wrong skin care, nutrition) are for these patients as relevant as endogenous ones (e.g., psychological stress, endogenous eruption, predisposition to dry, xerotic skin).[9–11] The atopic individual describes his skin in the symptom free intervals as a sensitive skin, which can be transformed by a combination of the mentioned triggers to a clinical visible atopic eczema. Indeed, many patients with acute eczematous problems complain about sensitive skin. This is so far understandable, since all kind of eczema are frequently accompanied by a skin barrier disruption leading to the so-called sensitive skin, since due to this barrier disruption even slight irritations (handwashing) may lead to a clinical visible skin reaction (e.g., worsening of the underlying dermatitis).[12] Such a skin reaction may concern individuals with rosacea, irritant dermatitis, nummular eczema, and exsiccation eczema as well.[13]

Other groups complaining about sensitive skin are subjects with nonvisible skin changes, or with a normal unimpaired skin. But, these individuals claim about skin symptoms after otherwise harmless affections of the skin, like the use of cosmetics, sun, wind, and clothes. However, some claim about sensitive skin even without any known about exogenous influences (Table 10.1).

Hence, a closer definition of the term sensitive skin is needed to avoid the mix-up of different entities of skin diseases and skin nondiseases.[14] The first group with sensitive skin indeed shows clinical signs, which can be detected by visual evaluation or by measurement of skin physiological parameters. Their complain concern all manifestations (clinical signs) of dermatitis and can be accompanied by any of the symptoms (Table 10.1). However, there is also a group complaining about sensitive skin without any clinical detectable skin changes (only the second row of Table 10.1).

10.1 A SELF-REPORTED SENSITIVE SKIN

The explanation for the visible symptoms in a group claiming about sensitive skin is founded in their skin precondition (e.g., dermatitis) and is mostly manifested in any degree of skin irritation. While nonvisible symptoms of other group are hard to explain, they are important for epidemiology: The number of individuals stating their skin as "sensitive" is amazingly high and is estimated at 50% with a clear dominance of women.[15] A very common statement of these individuals is that they have discomfort when using some cosmetic products.[15] It is anyhow very hard to verify such complaints using objective reproducible methods. Recently, we investigated whether sensitive skin is a result of a different anatomic or biophysical skin conditions (which can be evaluated by bioengineering methods) or, perhaps, the consequence of a different perception of skin sensations.[16]

We found in a questionnaire dealing with various influencing factors concerning skin susceptibility and skin sensitivity, which was completed by 420 volunteers, that in accordance to the study of Willis,[15] almost 50% of the volunteers estimated their skin sensitivity as strong or severe. However, the reason for their sensitive skin is hard to define. One reason may be an atopic constitution.

Indeed, we found a significant correlation between sensitive skin and atopy score. Hence, the subgroup of atopic patients is included in the group of volunteers with sensitive skin to a great extent. The majority of the volunteers with a sensitive skin had, however, no atopic constitution. But nearly any possible "trigger" such as sheep wool,

TABLE 10.1
Signs and Symptoms of Sensitive Skin

Clinical Signs of Sensitive Skin	Individual Symptoms of Sensitive Skin
Erythema	Itching
Xerosis	Burning
Infiltration	Tickling
Rhagaden	Pain
Papules	Tightening
Vesicules	Smarting
Oozing	
Erosion	
Excoriations	
Lichenifications	
Hyperkeratosis	

cosmetics, soaps, deodorants, perfume, or sun rays are suspected to worsen their sensitive skin. Interestingly, the skin susceptibility to sun rays was told to be enhanced, but there was no correlation to the skin type according to Fitzpatrick. It seems that, without any differentiation, every proposed influence was chosen by people with a self reported sensitive skin, however, the objectivability is missing.

Mostly, subjective terms were used to describe the problem with sensitive skin like the feeling of tension, burning, or reddening. Exactly defined and objectivable skin problems, like eczema, were not associated with the degree of sensitive skin. This again underlines the diversity between subjects with visible symptoms, like eczema, and patients with subjective nonvisible skin problems. This is supported by other investigators who also found a poor correlation between objective skin findings and subjective complaints about skin symptoms.[17]

In accordance to these findings, no changes in biophysical functions have been found in the group with the sensitive skin, neither basal nor after sodium lauryl sulfate (SLS) testing. In general, there was no correlation between the degree of self-estimated skin sensitivity and every single measured parameter. It therefore seems that the "sensitive skin" is a nonobjectivable estimation, probably influenced by the individual education and even more by the mass media. Furthermore, it seems indeed to be fashionable to have a sensitive skin, particularly for women and men in "modern society."[17]

These findings could be backed up by a study of Aramaki and co-workers. Aramaki et al.[18] investigated a population, which is known to have a very sensitive skin, namely Japanese individuals. Aramaki found that skin irritability tested by a SLS irritating test is the same between Germans and Japanese, but in a stinging test one could observe that the Japanese felt stinging immediately, whereas German women felt stinging somewhat later. The reason for these findings may be due to the assumption that Asian skin is more permeable to water than European skin and that several chemicals, like benzoic acid, caffeine, and acetylsalicylic acid showed an increase in percutaneous absorption in Asians relative to Caucasians,[19] but we also know that there is a certain difference in the culture and education between Japan and Germany. In Japan, the femininity is more desirable for women than in western countries, leading to a desirable, vulnerable, and female behavior. In this, the female role expectation of a dainty woman is expected to have a sensitive skin with problems with many cosmetics. However, it is hard to say whether the sensitive skin of Japanese women is caused by different cultural assessment of discomfort or by differences in penetration speed.

Even in these considerations about sensitive skin in Japanese and German women, the mix-up between different meanings of sensitive skin has become obvious. Is sensitive skin objective or subjective? Which parameters will define it? Hence, a better definition of all kinds of skin susceptibility is needed. The higher incidence of adverse skin effects against cosmetics is probably founded in a different perception of the skin.

Although previously changes in biophysical skin functions in the elderly have been found,[20] there was no correlation between complaints about sensitive skin and age of the subjects. This indicates, that the estimation of a sensitive skin is a complaint independent of age.

However, there are certain arguments that a sensitive skin does exist. One is the finding that individuals with a sensitive skin might be detectable by a stinging test and rather not by a skin irritation test. In contrast, there are lots of individuals who state their skin as sensitive but react normal in the stinging test and SLS patch test.

10.2 PROBLEMS WITH ENHANCED SKIN SUSCEPTIBILITY

Another group, which is particularly affected by the problem of sensitive skin, are patients who have a disrupted epidermal barrier function, for example, atopic patients or other groups with chronic eczematous skin. This group is likely to develop further eczema, often contact allergies.

In earlier years there was the impression that an allergic contact dermatitis could be caused by minimal doses of allergens. However, it has been shown that not only in the elicitation but also in the sensitization process, the concentration of the allergen per area is a crucial point. White et al.[21] have shown that the sensitization to dinitrochlorobenzene (DNCB), a potent sensitizer, only occurs, when a defined concentration threshold is reached. The explanation for this phenomenon was seen in the irritant feature of DNCB. Only if DNCB was applied in a concentration that a distinct irritant reaction was achieved, the sensitization process could take place. This irritation is therefore accepted as the "danger" signal necessary for each sensitization.[22,23] Mostly, the hapten itself can induce this danger signal because we know that almost every known relevant allergen is also an irritant. But if the hapten has a very low irritability, high concentrations are necessary for the sensitization. If lower concentrations are applied, the danger signal can be induced by additional substances. Experimentally, it has been shown that the vehicle of the hapten can induce this danger signal, so that the hapten itself does not necessarily have an irritant activity.[24] The coadministration of, for example, SLS,

a model irritant, with a hapten increases the chances of successful sensitization. Furthermore, when DNCB in a very low concentration is applied for sensitization, the accumulation of Langerhans cells in the draining lymph node and the proliferative response by draining lymph node cells can be increased by coadministration of the irritant SLS. This raised immunological activity can be compared to the one induced by DNCB alone in higher concentrations.[25] It can be assumed, that in these higher concentrations the irritant feature of DNCB is sufficient enough to induce a strong immunological reaction.

In metals, the coadministration of an irritant plays an important role, too. By the various attempts to sensitize mice against the common allergen nickel, it is known, that a simple injection or application of $Ni(II)Cl_2$ does not induce sensitization.[26] However, when applied together with an irritant, the experimental sensitization to $Ni(II)Cl_2$ can be successful.[27] A possible explanation for this observation is that the Ni-ion is altered in an inflamed skin. Phagocytes, which are activated by irritants produce oxidants like hypochloride (OCl^-) and hydrogen peroxide (H_2O_2), which are able to generate Ni(III) and Ni(IV) from Ni(II).[28,29] In this higher oxidation state, nickel contains a far higher chemical reactivity and it becomes able to sensitize naive T cells. Hence, the inflammation induced by an irritant does alter the nickel–hapten, so that a sensitization is possible. The same mechanism can be found for gold. When gold (I)—a weak sensitizer—is oxidized to gold (III), the sensitization capacity is raised enormously.[30,31] The impact on oxidation to the sensitizing capacity of allergens is generally known. Especially the dramatic effect of air-oxidation to various allergens (terpenes, fragrances, ethoxylated surfactants) has been investigated over the past years.[32–34]

But which cells are responsible for the initiation of the danger signal?

Today, the keratinocytes are in the focus of attention. It has been shown, that after stimulation by irritants (like SLS, DMSO, croton oil, and phorbol myristate acetate) keratinocytes produce a number of cytokines, for example, TNF-alpha, IL1-alpha, IL1-beta, IL-8.[35–39] But even the irritant property of Nickel is sufficient enough to induce such a cytokine release by keratinocytes.[40] TNF-alpha does of course induce an unspecific proinflammatory response by activation of T cells, macrophages, and granulocytes; it activates the expression of cellular adhesion molecules and the release of further cytokines.[41] More important in our context is the ability to activate Langerhans cells. TNF-alpha down-regulates E cadherin and induces the production of type-IV collagenase (MMP-9), so that the Langerhans cells can migrate more easily in the local lymph node.[42,43]

Moreover, TNF-alpha induces the up-regulation of MHC class-I and -II molecules, increasing the possibility of presentation of an allergen. As a consequence, TNF receptor p75 knockout mice are hardly sensitizable,[44] and anti-TNF-alpha antibodies or recombinant soluble receptors were able to block the sensitization.[45,46] Besides the known IL1-beta production (which is induced by irritants), the TNF-alpha production by keratinocytes seems to be a crucial point in the activation of the Langerhans cell migration,[47] and therefore in the concept of a danger signal (Table 10.2). An interesting theory is that in the absence of such a danger signal, the contact of the allergen with immunologic competent cells might induce tolerance.[21,22,48,49] This absence can be achieved by a very low dose of the allergen, so that the irritancy of the allergen is too small to induce any unspecific reaction, while the immunologic (allergenic) effect is still present. At this moment, the immunologic reaction might lead to tolerance. Experimentally, this was shown in mice by various groups;[50–53] the confirmation in humans is still missing.

In principle, the same danger signal may be necessary for elicitation and sensitization. Even the cytokine profile apparent at sensitization is very similar to that at elicitation; TNF-alpha and IL-1 are of highest relevance.[54] Often, the irritant property of the allergen may be sufficient enough to induce this danger signal. But if the allergen is applied on nonirritated skin in a concentration that no irritant reaction is induced, no allergic reaction takes place. The elicitation of an allergic response can then be achieved by coadministration of an irritant,[54] like SLS.[55,56] For the clinical practice, it is of highest relevance that in an irritated skin, the danger signal is already present. It means that a hapten does not have to induce the danger signal in the skin and therefore even a much lower concentration of the hapten is necessary for the sensitization and elicitation of an allergic dermatitis. Hence, especially patients with an irritant or atopic dermatitis have a much higher risk of developing a further skin sensitization and an additional allergic contact dermatitis. This association was assumed by epidemiological studies.[57,58]

For individuals with an enhanced skin susceptibility (more precisely, with an irritable skin), which is apparent as recurrent eczematous reactions, this danger signal–hypothesis is of highest relevance. They are in danger to develop more and more sensitizations during the course of an eczematous skin reaction, because once the reaction is induced, the danger signal is present due to the persistent inflammation. Further contact to the allergen may maintain this reaction, even when the concentration of the relevant allergen is below the concentration, which would normally be needed for the elicitation of the reaction (Table 10.2).

TABLE 10.2
Danger Signal and Its Cellular Mechanism

Danger Signals for Initiation of an Allergy: Irritation	Cellular Mechanism
High concentration of an allergen[21]	Up-regulation of MCH I and II, increased possibility of presenting an allergen[44]
Coadministration of an irritant[27]	Unspecific inflammation
Oxidation of metals[30,31]	Increased accumulation and activation of LCs in the draining lymph node[42,43]
Preirritated skin and epidermal disruption	Keratinocytes producing TNF alpha, IL1-beta, IL-8[36–39,59]

TABLE 10.3
Definition of Terms Concerning Skin Susceptibility

Term	Definition	Findings
Sensitive skin	Individuals, who stated their skin as more sensitive than average	None
Irritable skin	Individuals, who objectively develop a stronger skin reaction to an allergen than average	Enhanced values of bioengineering evaluation methods
Stinger	Individuals, who react reproducible positive in a test with a sensation inducing chemical	Reproducible positive skin stinging-test

10.3 DEFINITION OF TERMS CONCERNING SKIN SUSCEPTIBILITY

Concerning the various discussed mechanisms of sensitive skin, it becomes obvious, that the term sensitive skin has to be used very differentiated.

We propose the following classification of skin irritancy (Table 10.3):

People with sensitive skin are only individuals who stated their skin as sensitive. There is no possibility to prove the statement with objective methods, because the skin may have normal biophysiological skin parameter.

If such individuals react repeatedly to a skin test with sensation induced by chemical irritants (like lactic acid), they are identified as "stinger." It can be assumed that these individuals have indeed an increase in unpleasant skin sensations after use of otherwise well-tolerated skin products.

Moreover if individuals do have a high susceptibility to allergens or irritants because of a pre-irritated skin, which can be measured objectively by bioengeneering methods, they can be identified as individuals with an irritable skin.

This classification might be of relevance in discriminating individuals with a higher risk to develop skin irritation, particularly on the hands.[7,60–62]

With this classification it can be stated that there is neither any significant correlation nor any significant coincidence between individuals with a sensitive skin and individuals with an "irritable skin." Only a minority of individuals with a sensitive skin or an irritable skin can be identified as stingers.[63–66] Hence, if the patient is stating, "I have a very sensitive skin," he does not give you any important information, but solely telling you that he takes a lot of care of his skin.

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11 Transdermal Drug Delivery System: An Overview

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11.1 INTRODUCTION

The skin is a seemingly impermeable barrier with its primary function to protect against entry of foreign agents into the body. Nevertheless, intact skin may be used as a route of administration for systemic delivery of simple potent drug molecules through a transdermal patch. In fact, recent literature suggests that the transdermal route now competes with oral administration as the most successful innovative research area in drug delivery, as 40% of current drug products under clinical evaluation are transdermal (Barry, 2001). The first U.S.-approved transdermal patch was introduced in 1981 for scopolamine. In the past two decades, another nine drugs have been introduced to the U.S. market, namely nitroglycerin and clonidine for cardiovascular disease, nicotine for smoking cessation, fentanyl for chronic pain, estradiol with or without levonorgesterel or norethisterone for hormone replacement, estradiol with norelgestromin for hormonal contraception, testosterone for hypogonadism (Hogan and Cottam, 1991), lidocaine for postherpetic neuralgia, and oxybutynin for detrusor hyperactivity, and most recently selegiline to treat major depressive disorder. Many more transdermal drug delivery systems (TDDS) are currently under investigation, including products to treat Parkinson's disease, Alzheimer's disease, and skin cancer (Benson, 2005) (Table 11.1).

11.2 PERCUTANEOUS DRUG ABSORPTION

The skin's uppermost layer of epithelium, the stratum corneum (SC), is the rate-limiting barrier to percutaneous drug transport. In fact, it is significantly more impermeable than the gastrointestinal, vaginal, nasal, buccal, or rectal epithelial barriers. Consequently, the daily dose of drug that can be delivered is only 5–10 mg, thereby limiting this route to the most potent of drugs. Drug molecules may penetrate the SC through three potential pathways: directly across the SC, through the sweat ducts, or via the hair follicles and sebaceous glands (also known as the appendages). Prior experimentation has suggested that the follicular route comprises only 1/1000 of the entire skin surface area, and therefore most skin penetration systems focus on enhancing penetration directly across the SC.

The SC is comprised of a multilayered brick and mortar structure of keratin-rich corneocytes in an intracellular matrix of fats, including long chain ceramides, free fatty acids, triglycerides, cholesterol, and sterol/wax esters. It was traditionally thought that hydrophilic chemicals diffuse within the aqueous regions near the outer surface of intracellular keratin filaments while the lipophilic chemicals diffuse through the intercellular routes, through the lipid matrix between the filaments. This is a gross oversimplification, and more recent data suggest that the intercellular route is the major pathway for

TABLE 11.1
Transdermal Delivery Systems

Active Drug	Duration of Application	Matrix or Membrane	Typical Formulations
Nitroglycerin	12–24 h (depending upon brand)	Matrix	0.1, 0.2, 0.4, 0.6, or 0.8 mg/h
Clonidine	1 week	Membrane	0.1, 0.2, or 0.3 mg/day
Nicotine	24 or 16 h	Membrane or matrix	21, 14, or 7 mg/day or 15 mg in 16 h
Fentanyl	72 h	Membrane	1.25, 2.5, 5, 7.5, or 10 mg/h
Estradiol	3 days to 1 week (depending upon brand)	Matrix	0.025, 0.0374, 0.05, 0.075, or 0.1 mg
Estradiol/levonorgestrel	1 week	Matrix	0.045 mg estradiol and 0.015 mg levonorgestrel per day
Estradiol/norethindrone acetate (NETA)	3–4 days	Matrix	0.05 mg estradiol and between 0.14 and 0.25 mg NETA per day
Norelgestromin/ethinyl estradiol (EE)	7 days	Matrix	150 mcg norelgestromin, 20 mcg EE per day
Testosterone	24 h	Membrane	2.5–5 mg/day
Lidocaine	12 h, usually three patches at a time	Matrix	Three patches yield 64 ± 32 mg/12 h
Oxybutynin	3–4 days	Matrix	3.9 mg/day
Selegiline	24 h	Matrix	6 or 9 or 12 mg selegiline in 24 h

Source: *Physicians Desk Reference*, 2006.

both lipophilic and hydrophilic chemicals. Therefore, most penetration enhancement techniques aim to alter the structure or manipulate the solubility of the lipid domain (Benson, 2005; Elias and Friend, 1975; Bodde, 1991).

Once a drug is able to cross the SC, it is relatively easy to permeate the deeper epidermal and dermal layers and become systemically absorbed. The epidermis is comprised of cells with a greater degree of hydration allowing faster diffusion of drug. In addition, Langerhans cells, the antigen-presenting cells of the skin, present in the epidermis are the target of transdermal vaccines. Small vessels in the dermis allow for distribution of drug to the systemic circulation. The lymphatic network in the dermis is responsible for removal of transdermally applied drugs, although this has not been studied extensively.

The challenge in transdermal drug delivery is systemic absorption in a safe, controllable, and therapeutic fashion without permanently reducing the efficacy of the skin barrier (Berti and Lipsky, 1995). Rate and extent of drug absorption must be tightly controlled to successfully achieve these goals. Factors such as the thickness of the SC in a body region (Ya-Xian et al., 1999) (SC is thicker on the palmar and plantar regions and thinner on the postauricular, axillary, and scalp) and formulation of vehicle are instrumental in designing the appropriate patch. In general, drugs that have been successfully designed for a transdermal system are small, of low molecular weight, high potency, and of moderate lipophilicity (because they must get through the hydrophilic epithelial and dermal layers) (Ogiso and Tanino, 2000; Kalia and Guy, 2001).

11.3 TRANSDERMAL DRUG DELIVERY DEVICES

There are currently two types of transdermal drug delivery devices—matrix-controlled and membrane (reservoir)-controlled (Figures 11.1 and 11.2) (Ranade, 1991). The matrix-controlled device incorporates a drug-in-polymer matrix layer between frontal and backing layers, where the matrix binds to the drug and controls its rate of release from the skin. In the reservoir system, there is a rate-controlling membrane present between the drug matrix and the adhesive layer, which controls the rate of drug release. In both systems, the rate of drug permeation is greater than the permeation rate across the skin. This provides drug uptake at a predetermined rate that is independent of patient skin variability (Ansel and Allen, 1999).

The advantage of the membrane-controlled system is that it provides a true constant (zero-order) release of drug from the system, irrespective of the amount of drug remaining in the patch. In the matrix system, the rate of release is dependent on the matrix bound to the drug. As drug is depleted from the system, there is a slight decline in the release rate when using the matrix system. This is because drug in the surface layers has already permeated and the remaining drug must diffuse a longer distance through the matrix to penetrate the skin. With well-designed matrix systems this rate is insignificant. A disadvantage of the reservoir system is that drug molecules may saturate the rate-controlling membrane and thereby cause a "burst effect," whereby the patch initially releases too much drug into the system and potentially causes toxicity. Of course, the burst effect may be advantageous for drugs that normally exhibit a long lag time between patch application and therapeutic effect (Ranade and Hollinger, 1996; Kydonieus, 1992).

11.3.1 Advantages of Transdermal Drug Delivery

Transdermal drug delivery avoids the gastrointestinal and hepatic first-pass metabolism and thereby increases drug

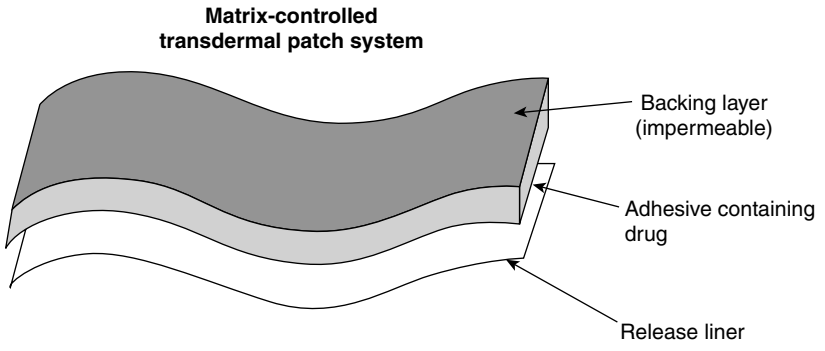


FIGURE 11.1 Matrix-controlled device.

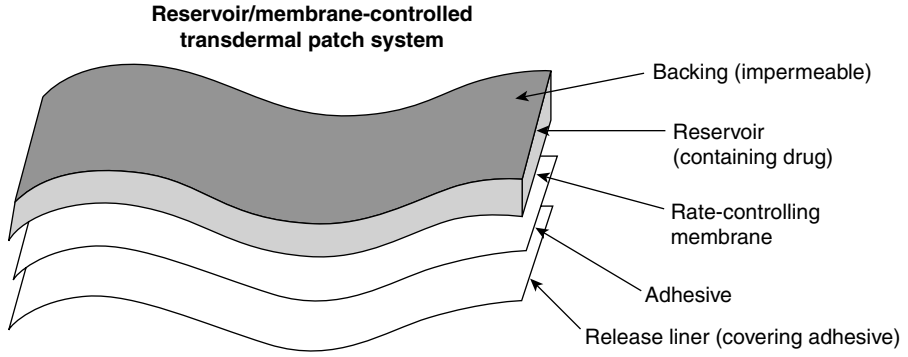


FIGURE 11.2 Membrane-controlled device.

bioavailability in comparison to oral formulations. Another important advantage of the TDDS is the elimination of the generally observed "peaks" and "valleys" in the plasma drug concentration profile observed in patients receiving oral drug delivery formulations (Kydonieus, 1992). With the appropriate TDDDS the drug input rate into the bloodstream can be controlled to stay in the therapeutic region, potentially avoiding toxic or sub-therapeutic drug plasma levels. In addition, transdermal systems also reduce the frequency of drug administration and some allow for multiday continuous drug delivery. These factors may improve patient compliance (Bronaugh and Maibach, 1999).

11.3.2 Disadvantages of Transdermal Drug Delivery

The main disadvantage of the use of transdermal drug delivery is in its limitations. The SC is an excellent barrier and therefore only small; highly potent molecules with moderate lipophilicity will be candidates for transdermal delivery (Bronaugh and Maibach, 1999). In addition, there is the potential for a long lag time between patch application and effect of drug, which is a disadvantage when immediate drug action is needed. Depending upon the circumstance, it may be necessary to supplement with oral medication during the initial patch application. Other disadvantages include variable intra- and interindividual percutaneous absorption efficiency, variable adhesion to different skin types, and a limited time that the patch can be affixed. Finally, TDDS are currently only developed when conventional administration has serious limitations. This is because transdermal systems are costly and involve complex technology to develop.

11.4 VARIABLE INTRA- AND INTERINDIVIDUAL PERCUTANEOUS ABSORPTION EFFICIENCY

Transdermal systems are designed to account for intra- and interindividual differences that affect the percutaneous absorption of the drug. Interindividual differences include age and ethnicity. It is well established that the skin of neonates and the elderly is more permeable than middle-aged adults. Additionally, there is evidence to suggest that Caucasian skin is more permeable than black skin (Astner, 2006). Intraindividual differences include body site and skin status or condition. The scrotal skin, mucous membranes, and eyelids are the most permeable to drugs, while the chest/back, buttocks, and upper arms/legs are areas of relative impermanence. Other skin conditions that increase permeability include hydrated skin, irritated or broken skin, thermal burns, warmer skin, and eczematous skin (Wikosz, 2003). To minimize such potential differences, transdermal manufacturers will specify the skin site, duration of time, and age range for which they recommend using their product.

11.5 ADVERSE REACTIONS TO TRANSDERMAL SYSTEMS: GENERAL

The adverse reactions due to transdermal systems may be classified into two broad categories: local irritation due to the patch itself or a systemic and immunologic reaction to the components of the patch (Maibach, 1992).

11.5.1 Local Effects

11.5.1.1 Occlusion

TDDS are occlusively applied to the skin for 1–7 days (Brown and Langer, 1988). Occlusion enhances skin hydration by allowing eccrine sweat glands and water vapor to accumulate on the skin's surface (Hurkmans et al., 1985). Hydration not only enhances skin absorption of compound but may also lead to adverse effects, such as miliaria rubra, caused by sweat duct occlusion. In miliaria rubra, sweat infiltrates below the epidermis and results in pruritic erythematous papulovesicles (Hurkmans et al., 1985). Generally, the miliaria rubra is limited to the application site and is resolved within 1 day. Treatments for miliaria rubra include topical steroids to reduce the associated itching, and increased fluid intake to maintain homeostasis in the body. To prevent miliaria from occurring, transdermal patches are not reapplied to the same site following a 1–7 day course. Transdermals should not be given for more than 7 days at a time.

11.5.1.2 Erythema

Transient mild to moderate erythema occurs in most patients. Removal of the pressure-sensitive adhesive is responsible for the erythematous skin response (Fisher, 1984).

11.5.1.3 Irritant Contact Dermatitis

There is an increased risk of developing irritant contact dermatitis (ICD) proportional to the increased occlusive period of the patch. Additionally, irritant dermatitis may occur if the transdermal system is repeatedly applied to the same skin site.

11.5.1.4 Miscellaneous

Scopolamine patches have been implicated in causing anisocoria in several patients when they are inadvertently transferred to another site (Carlston, 1982; McCrary and Webb, 1982). A nitroglycerin disk was implicated in causing a second-degree burn on a patient's chest, in one case report (Murray, 1984). Person-to-person transfer of transdermal therapeutic systems may cause toxicity in the recipient (Wick et al., 1989). This is most likely to occur when the TTD has been applied for a long time to one site. The longer the application period of the TDDS, the lower the skin adhesion and the more likely the system will become dislocated inadvertently.

11.6 SYSTEMIC/IMMUNOLOGIC

The most common type of hypersensitivity reaction to the TDDS is an allergic contact dermatitis. Generally, the reaction is in response to a component of the drug or the adhesive background in the transdermal system (Nieboer et al., 1987); the reaction is prevalent during any stage of drug use. Lanolin, a vehicular ingredient in many of the transdermal ointments, is one of the primary accountable agents. Patches applied to thin-skinned areas, such as the postauricular region (scopolamine) or the scrotal area (testosterone) are more likely to cause ACD. Sensitization is also promoted in response to local irritation (resulting from prolonged occlusion) as well as oily, inflamed, broken, calloused or occluded (Dwyer and Forsyth, 1994; Nieboer et al., 1987) skin. Lack of patient compliance with regard to applying the patch to the designated site, applying the patch for a designated period of time, or rotating patch application sites may also lead to skin sensitization. The skin hypersensitivity reaction is generally manifested as a rash accompanied by redness, burning, itching, heat, and swelling. If the reaction persists, the therapeutic system should be discontinued.

11.7 PROPHYLACTIC MEASURES TO DECREASE ACD INCIDENCE

Allergic contact dermatitis from TDDS is one of the limiting factors in patient compliance. Some prophylactic measures that should/are being taken to decrease the incidence of ACD include limiting patch application time and rotating the patch site. Improved predictive tests of sensitization should be developed for each of the therapeutic systems. In the development of clonidine, standard predictive tests indicated that it was not an allergen; however, when marketed, many users developed allergic contact dermatitis. Currently, mouse strains are being developed as an alternative to the standard guinea pig model (Kalish et al., 1996). Robinson and Cruze used guinea pig models and a local lymph node assay (LLNA) in mouse models to aid in the detection of weak allergens (Robinson and Sozeri, 1990). Through vitamin A supplementation and the introduction of chronic conditions, they were able to detect contact sensitization of clonidine in both mice and guinea pigs. Other predictive models have analyzed the electrophiles within various haptens, such as scopolamine and clonidine and their relationship with nucleophilic groups of skin protein to form antigens (Benezra, 1991). Contact sensitization from transdermal *d*-chlorpheniramine and benzoyl peroxide was reduced when hydrocortisone was coadministered (Amkraut et al., 1996). Additionally, when applied prior to the transdermal patch, ion channel modulators, such as ethacrynic acid, have been found to prevent sensitization in mice (Kalish et al., 1997; Wille et al., 1999). The potential role of corticosteroids and ion channel modulators in the prevention of contact sensitization from Transdermal therapeutic system (TTS) should be further investigated and better defined. Additionally, minimizing reapplication to the same application site (Hogan and Cottam, 1991) and maintaining caution when performing oral provocation tests (Vermeer, 1991) would help to reduce the induction of allergic contact dermatitis among TTS users.

11.8 ACTIVE TRANSDERMAL DRUG DELIVERY AND PENETRATION ENHANCERS

The advantages associated with TTS, including avoidance of the first-pass metabolism, elimination of "peaks and valleys," and improved patient compliance, have led to continual interest

in the development of new transdermal systems. Some transdermal systems that may be available in the United States in the near future include insulin (Sen et al., 2002) and asthma (Kato et al., 2002) medication.

The current transdermal delivery systems are useful for delivering small, lipophilic molecules through the skin. However, there are many compounds that do not meet these requirements. New techniques are being developed to allow the transfer of hydrophilic, charged drugs through the skin. "Active" transdermal drug delivery involves utilizing external driving forces on the SC to allow penetration of the molecule of interest (Barry, 2001).

Electrically assisted methods include iontophoresis, phonophoresis, electroporation, magnetophoresis, and photochemical waves. Iontophoresis passes an electric current through the skin and thereby provides the driving force to enable penetration of ions into the skin (Singh et al., 1995). The development of transdermal insulin (Rastogi and Singh, 2002), dexamethasone (Nirschl, 2003), naproxen (Baskurt, 2003), and others may require the use of iontophoresis. Phonophoresis utilizes ultrasound energy to enhance drug penetration. Higher-frequency energy not only enables greater penetration but is also associated with greater adverse events. Electroporation uses strong, brief pulses of electric current to punch holes in the SC. These holes close 1–30 min following the electrical stimulus (Banga and Prausntiz, 1998). Electroporation coupled with iontophoresis may be helpful in the delivery of some drugs (Badkar et al., 1999). This technique is currently being studied to eventually allow transdermal delivery of drugs such as metoprolol (Vanbever, 1994) and heparin (Prausnitz, 1995). The application of high-gradient magnetic fields and vibrational forces to biological systems is termed magnetophoresis. Magnetophoresis may be effective in the delivery of terbutaline sulfate (TS), a drug widely used for the treatment of acute and chronic bronchitis patients (Narasimha and Shobha Rani, 1999). Laser-induced stress waves, known as photochemical waves, may also benefit drug delivery. More recently, ultrasound or sonophoresis has been studied *in vitro* to increase the permeability of drugs such as insulin and aldosterone (Mitragotri, 2001).

Hydrating agents and chemical enhancers (Smith and Maibach, 1995) also increase pore size to enhance drug delivery. Moisturizers are the primary hydrating agents. There are numerous chemical enhancers, including benzalkonium chloride, oleyl alcohol, and alphaterpineol (Monti et al., 2001; Sinha and Kaur, 2000). Transdermal delivery may be most improved by utilizing a combination of chemical enhancers and electrically assisted devices (Terahara et al., 2002).

Structurally based techniques have recently been developed to actively increase drug permeability. The SC may be removed or bypassed utilizing ablation and thus increasing drug permeability. Microneedles and jet-propelled particles are two relatively new techniques for physically increasing drug permeability. Microneedles are used to create a physical pathway through the upper epidermis and thereby increases skin permeability. The needles are approximately 150 mcm length and 80 mcm diameter, and are fabricated onto arrays. The needles penetrate the SC and epidermis without reaching nerve endings present in the upper dermis and are thereby painless. Recent experimentation suggests that this technology has allowed greater than fourfold increase in drug permeability (Figure 11.3).

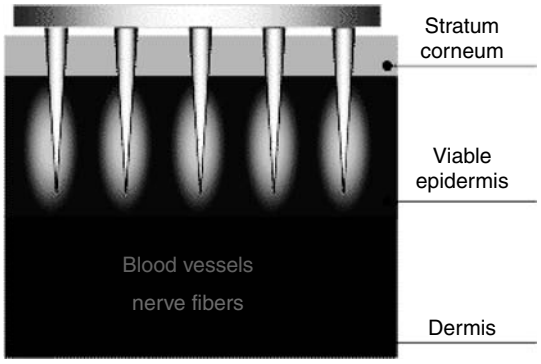


FIGURE 11.3 Microneedle technology.

The transdermal jet-injectors propel drug molecules into the skin through the production of a high-velocity jet of compressed gas (usually helium) that accelerates through the nozzle of the injector device, carrying its drug particles. The injectors are currently being studied for the injection of macromolecules, as well as in the delivery of DNA or protein vaccines into the epidermis (Cross, 2004; Dean, 2003).

Although passive transdermal systems have been on the market for more than 20 years, active transdermal systems are still not available for clinical use. In the next few years, we await the development of new passive transdermal products and the onset of clinical studies utilizing active transdermal techniques to promote drug permeability.

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12 Iontophoresis: From Historical Perspective to Its Place in Modern Medicine

Angela N. Anigbogu and Howard I. Maibach

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12.1 INTRODUCTION AND HISTORICAL PERSPECTIVES

The skin is the largest organ in the human body and has long been used as a site for administration of therapeutic agents for localized pharmacological actions (Kastrip and Boyd, 1983). Drug delivery through the skin for systemic effects, though limited, is a well-established branch of pharmaceutics. The stratum corneum, the outermost layer of the skin offers excellent barrier properties to applied substances thus limiting the number of drug candidates for passive transdermal delivery to usually small, potent, and lipophilic compounds. Physical and chemical techniques have been used to improve the permeability of the skin to applied substances. Dermal iontophoresis is one of such physical techniques.

A Greek physician, Aetius, first prescribed shock from electric fish for the treatment of gout more than 1000 years ago and since then the use of electric current to introduce drugs into the body has intrigued scientists. Iontophoresis was first introduced by Pivati to treat arthritis in the 1740s (Licht, 1983) and Palaprat claimed in 1833 to have been able to deliver iodine directly to tissues by means of electric current (Jones, 1907).

Iontophoresis may be defined as the facilitated transport of ions of water-soluble salts across membranes under the influence of an applied electric field. The technique temporarily lost its importance partly because it was not well understood and partly due to safety considerations. Earlier, Munch demonstrated the systemic application of this technique in 1879, when strychnine delivered under the positive electrode in

rabbit killed the animal within 15 min of current passage. Leduc (1900) described some of the earliest systematic experiments outlining the usefulness of iontophoresis in systemic drug delivery. He placed a solution of strychnine sulfate (positively charged strychnine ion) in the positive electrode (anode) of an iontophoresis setup on one rabbit with the negative electrode filled with water and a solution of potassium cyanide (negatively charged cyanide ion) in the negative electrode (cathode) of a setup on another rabbit with the positive electrode filled with water. The animals were connected and when a constant current of 40–50 mA was applied, both animals died due to strychnine and cyanide poisoning, respectively. In a subsequent experiment, reversing the polarity of the delivery electrodes (i.e., strychnine in the cathode and cyanide in the anode), neither animal died demonstrating that in the first case, the electric current delivered the lethal ions.

Since the early years, there has been a resurgence of interest in iontophoresis. Gibson and Cooke (1959) used iontophoretic delivery of pilocarpine to induce sweating and the procedure is now used for the diagnosis of cystic fibrosis, a method still used in modern medicine for the routine diagnosis of cystic fibrosis even in children and neonates (Santos et al., 2005; Ahn et al., 2005; Mackay et al., 2006). Iontophoresis has been used for the treatment of palmoplantar hyperhydrosis. In addition to this and other local applications of the technique, the present focus of research and development efforts on iontophoresis is for systemic drug delivery. With interest in controlled drug delivery surfacing in the last two decades, and the inability to deliver a great number of drugs especially proteins and peptides passively, iontophoresis appears to be particularly attractive and holds great commercial promise for noninvasive rate-controlled transdermal drug delivery for a wide array of drugs including hydrophilic, charged, and high molecular weight compounds all of which would not permeate the skin by passive diffusion.

12.2 THEORY

Biological tissues including skin consist of membrane barriers made up of lipids and proteins. Transport through these membranes is better suited to unionized than ionized compounds. Many potential drug candidates are ionized at skin pH (4–5) and cannot therefore be transported across membranes passively. As stated previously, the stratum corneum provides an excellent barrier to transport across the skin. In addition, passive diffusion depends on a concentration gradient across the membrane. Membrane transport of drugs can be facilitated by the application of an external energy source (active transport).

Iontophoresis by utilizing electric current provides an excellent source of this external energy. It operates on the general principles of electricity, i.e., opposite charges attract and like charges repel. Thus, if the drug of interest is cationic, for delivery across the skin it is placed in the anode reservoir. When a voltage is applied, the positively charged drug is repelled from the anode through the skin and into the systemic circulation. Conversely, an anionic drug is placed in the cathode reservoir. The transport of polar neutral and uncharged molecules (Gangarosa et al., 1980; Sims and Higuchi, 1990; Banga, 1998; Bath et al., 2000) as well as high molecular weight cations (Pikal, 1992) can also be facilitated by iontophoresis by the process of current induced convective water flow also known as electroosmosis. Electroosmosis may in fact be the major underlying mechanism in iontophoretic delivery of proteins and peptides. In addition to the major mechanisms of electro-repulsion and electroosmosis, the application of electric current may itself directly increase passive diffusion across the skin by reversibly disorganizing skin lipids and proteins. Figure 12.1 is an illustration of an iontophoretic setup.

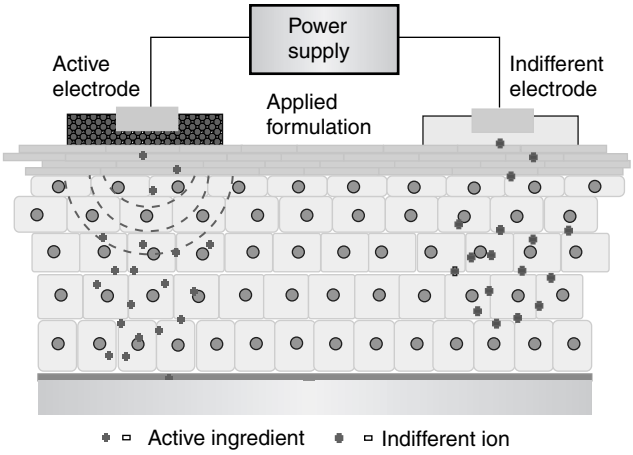


FIGURE 12.1 A schematic depiction of the basic principles of transdermal iontophoresis showing drug repulsion from the active electrode toward the indifferent electrode and into the skin upon passage of current. (From Daniels, R. Skin Care Forum, Online Issue 37, 7, 2004. With permission.)

In this section, the underlying principles of iontophoretic transport will be described briefly. The Nernst–Planck flux equation as applied in iontophoresis provides that the flux of an ion across a membrane under the influence of an applied charge is due to a combination of iontophoretic (electrical potential difference), diffusive (increased skin permeability induced by the applied field), and electroosmotic (current-induced water transport) components (Schultz, 1980).

$$J_{ion} = Je + Jp + Jc \tag{12.1}$$

where *Je* is the flux due to electrical potential difference and is given by:

$$Je = \frac{ZiDiF}{RT} Ci \frac{\partial E}{\partial x} \tag{12.2}$$

and *Jp* is the flux due to passive delivery and is given by:

$$Jp = KsDs \frac{\partial C}{\partial x} \tag{12.3}$$

and *Jc* is the flux due to electroosmosis or convective transport and is given by:

$$Jc = kCsI \tag{12.4}$$

where

Zi = valence of the ionic species
Di = diffusivity of the ionic species, i in the skin
F = Faraday constant
T = absolute temperature
R = gas constant
$\partial E, \partial x$ = electrical potential gradient across the skin
Ci = donor concentration of the ionic species
Ks = partition coefficient between donor solution and stratum corneum
Ds = diffusivity across the skin
$\partial C, \partial x$ = concentration gradient across the skin
Cs = concentration in the skin
I = current density
k = proportionality constant

(Chien et al., 1990).

In iontophoretic drug delivery, the major contribution to the overall flux of a compound would be due to electrical potential gradient (electromigration). The contribution to the flux due to electroosmosis is likely to be small (Srinivasan et al., 1989) and Roberts et al. (1990) have suggested that only about 5% of the overall flux is due to convective solvent flow. In the anodal iontophoresis of lidocaine hydrochloride, electromigration was shown to contribute approximately 90% of the total flux (Marro, et al., 2001a). Electroosmosis is always in the direction as the flow of the counterions. Human skin is negatively charged at pH above 4 and the counterions are positive ions and therefore, electroosmotic flow would occur from anode to cathode.

The Goldman constant field approximation is used to facilitate the integration of Equation 12.1 to give an enhancement factor E (relative to passive flux) which is given by (Srinivasan et al., 1989):

$$E = (Flux_{\text{ionto}} / Flux_{\text{pass}}) = \frac{-K}{1-\exp(K)} \tag{12.5}$$

where

$$K = \frac{ZiF\Delta E}{RT} \tag{12.6}$$

At high voltages, deviations from the predictions based on Equation 12.5 have been known to occur (Srinivasan et al., 1989; Kasting and Keister, 1989).

While the Nernst–Planck equation when applied to iontophoresis describes the flux of a drug through a membrane under the influence of applied potential, Faraday's law describes flux in terms of electric current flowing in the circuit. Applying Faraday's law therefore, the mass of substance transported in an aqueous solution is proportional to the charge applied (this is the product of current and time). The flux of a compound transported across the skin (J_{trans}) is thus given by:

$$J_{\text{trans}} = \frac{t}{zF} I \tag{12.7}$$

where

t = transport number of the compound
z = charge on the drug
F = Faraday's constant and is equal to 96,500 C/mole
I = current density (A/cm^2).

In iontophoresis, all ions in a formulation as well as ions in the skin carry a fraction of the applied electric current. The most important ions for consideration, are however, those of the drug of interest with transport number t, which is defined as the fraction of the total current carried by the drug and is given by:

$$t = \frac{z^2 mc}{\sum_i z_i^2 m_i c_i} \tag{12.8}$$

where

z = charge of the drug
m = ionic mobility
c = concentration of the ion and
i = all the ions in the system.

In theory, therefore, if the mobility of a drug in the skin is known, the iontophoretic flux can be predicted. In practice, however, it is not easy to estimate the skin mobility of a drug and free solution mobility is thus usually used as an approximation (Singh et al., 1997). The efficiency of iontophoretic drug delivery is therefore governed by both thermodynamics and electrochemistry being influenced by a combination of valency, polarity, mobility of the ionic species, formulation composition, and electrical duty cycle.

12.3 IONTOPHORESIS DEVICES AND EXPERIMENT PARAMETERS

12.3.1 *In vitro*

In the early stages of the revival of iontophoresis, relatively simply techniques were employed for the delivery of small molecules. Examples include Molitor (1943); Burnette and Marrero (1986); Bellantone et al. (1986); Masada et al. (1989); Green et al. (1991); Thysman et al. (1991); and Chang and Banga (1998). Usually these involve modifications of the two-compartment *in vitro* passive diffusion set-up. Two electrodes connected to a power supply are used, and in some instances, one is inserted in each compartment separated by the mounted skin and voltage or current measurements are made between the electrodes. In other instances using vertical flow through diffusion cells, a horizontally mounted

piece of skin separates the positive and negative electrode chambers with the epidermal side of the skin from the receptor phase bathing the dermal side.

A four-electrode potentiostat system designed to maintain a constant voltage drop across a membrane in a two-chamber diffusion cell has been described by Masada et al. (1989). As with passive diffusion studies, the whole assembly is kept at 37°C with the aid of a constant-temperature water bath to maintain the skin surface temperature at 32°C.

In all constructs, the receiver compartment medium is usually stirred with the aid of magnetic bar stirrers. The electrodes usually consist of platinum wires or silver/silver chloride. Cationic drugs are placed under the anode electrode in the donor compartment with the cathode in the receiver compartment and the opposite is true of anionic drugs. Pulsed or constant current may be applied.

Regardless of the type of electrodes and cells used, the same principles and transport mechanisms apply. Bellatone et al. (1986) demonstrated that diffusion cell type had little impact on the diffusion of benzoate ions across hairless mouse skin. Similarly, Kumar et al. (1992) have shown that cell design was not a factor in the delivery of an analog of growth hormone releasing factor *in vitro* across hairless guinea pig skin by iontophoresis.

Furthermore, iontophoresis has been combined *in vitro* with other enhancement techniques (physical or chemical) such as skin penetration enhancers as in skin pretreatment with oleic acid or oleic acid added to the drug formulation for the delivery of piroxicam (Gay et al., 1992); sodium lauryl sulfate or cetrimide to the donor in the delivery of Acyclovir (Lashmar and Manger, 1994); skin pretreatment with surfactant or a suspension of elastic liquid-state vesicles for the delivery of Apormorphine (Junginger, 2002); ethanol or a combination of terpenes and ethanol for the delivery of Insulin (Pillai and Panchagnula, 2003); terpenes for the delivery of Buspirone hydrochloride (Al-Khalili et al., 2003); lipid extraction for the delivery of insulin (Rastogi and Singh, 2002); electroporation for the delivery of Timolol and Atenolol (Denet et al., 2003) and 5-fluorouracil (Fang et al., 2004); laser treatment for the delivery of 5-fluorouracil (Fang et al., 2004); and liposomes and liposomes and electroporation in the delivery of estradiol (Essa et al., 2004).

12.3.2 *In vivo*

Devices used in iontophoresis are designed for rate-controlled delivery of therapeutic agents. The devices used *in vivo* vary in complexity from those that use household current to battery-and-rheostat type to modern electronic circuit devices (Singh and Maibach, 1993). Essentially, they consist of a power source to provide current, anode, and cathode reservoirs. The reservoir electrodes usually consist of a small metal plate over which a moist material preferably a pad or gauze is overlaid and this portion comes in direct contact with the skin. During use, an indifferent electrode (without drug) is placed some distance from the active electrode. Regardless of design, the most important considerations in choosing an iontophoretic device include safety and comfort of patients, cost, ease of operation, reliability, size, and therefore, portability.

Generally, they are operated at a constant voltage allowing the current to be varied for patient comfort and compliance over a given period. As with *in vitro* apparatuses, various devices have been described for use in iontophoresis *in vivo* (Molitor and Fernandez, 1939; Barner, 1961; Rapperport et al., 1965). Rattenbury and Worthy (1996) described systems used in the U.K. Hidrex (Gessellschaft für Medizin and Technik, Wuppertal, Germany) has been described by Hölzle and Alberti (1987). Phipps et al. (1989) described a custom-made battery operated device with two hydrogel electrodes for *in vivo* delivery of pyridostigmine. These devices deliver direct steady current, which have been postulated to be responsible for skin irritation arising from iontophoresis due to continuous electric polarization. To minimize this, others advocate devices delivering pulsed current such as have been used to administer catecholamines to dogs (Sanderson et al., 1987). In furtherance of this argument, two delivery systems using pulsed direct current have been described, one being the advance depolarizing pulse iontophoretic system (ADIS-4030) designed to continuously deliver drugs under constant pulsed current application (Okabe et al., 1986). The other, the transdermal periodic iontophoretic system (TPIS) delivers pulsed direct current with combinations of frequency, waveform, on/off ratio, and current density, for a programed treatment duration (Chien et al., 1990).

Available in the United States is a portable battery operated power supply unit called a Phoresor® (Dermion Drug Delivery Research, Salt Lake City, Utah, USA) and it is suitable for home use.

The US Food and Drug Administration has categorized iontophoretic devices into those for specialized uses (Class II) and others (Class III) (Tyle, 1986). These include Drionic® (General Medical Company, Los Angeles, CA, USA), Macroduct (Wescor Inc., Logan, UT, USA), Iontophor-PM (Life-Tech Inc., Houston, TX, USA), Model IPS-25 (Farrall Instruments Inc., Grand Island, NE, USA), Electro-Medicator (Medtherm Corporation, Huntrille, AL, USA), Dagan® (Dagan Corporation, MN, USA), and Desensitron II® (Parkell, Farmingdale, NY, USA).

12.4 CHOICE OF ELECTRODE MATERIALS IN IONTOPHORESIS

Platinum electrodes or patches consisting of zinc/zinc chloride or silver/silver chloride electrodes are used. The choice of electrode material depends on several factors including good conductivity, malleability, and the ability to maintain a stable pH. In addition, the electrodes should not produce gaseous by-products and must be safe to be used on the skin. Silver/silver chloride electrodes also referred to as reversible electrodes are made from a metal in contact with solution of its own ions (Boucsein, 1992) and are the most commonly

used as they satisfy these requirements. At the anode, silver under the influence of an applied electric field is oxidized and reacts with chloride to form silver chloride. At the cathode, silver chloride is reduced to silver with the liberation of chloride ions. These electrodes are thus stable. They should however be thoroughly cleaned after use and rinsed with distilled water. When not in use for prolonged periods, it is advisable to store the electrodes dry.

Platinum electrodes though used are less desirable in some situations than silver/silver chloride electrodes because with prolonged use of platinum electrodes, there is oxidation of water to oxygen gas and hydronium ions. This was demonstrated by Phipps et al. (1989) in anodal delivery of lithium across hydrogel membranes. The efficiency of delivery achieved using platinum electrodes was 20% compared to 37% when silver anode was used instead.

Careful selection of the electrode used to deliver a particular drug is also important. For instance, dexamethasone sodium phosphate can be delivered under the anode electrode by electroosmosis. However, considering that electroosmosis contributes a small fraction to the overall iontophoretic transport of any given drug, cathodal iontophoresis should therefore be considered. It is in fact known that the delivery efficiency of dexamethasone sodium phosphate by iontophoresis from the cathode is far greater than from the anode. It has been suggested that for monovalent ions with Stoke's radii larger than 1 nm, electroosmotic flow may be the dominant transport mechanism. In addition, for large anions or negatively charged protein, electroosmotic flow from the anode may be more efficient than cathodal electromigration (Pikal, 2001).

12.5 ANIMAL MODELS

The ultimate goal of any research done in the field of iontophoresis is the application in humans for drug delivery. For obvious reasons, animals and not human subjects are the first choice for experimental purposes. There is no consensus as to which of the animal models used in passive uptake studies is suitable for iontophoresis. Hairless mouse has been the most commonly used model (Bellantone et al., 1986). Other models that have been investigated include hairless guinea pig (Walberg, 1970), dog (McEvan-Jenkinson et al., 1974), furry rat (Siddiqui et al., 1987), pig (Monteiro-Riviere, 1990), hairless rat (Thysman and Preat, 1993), and rabbit (Lau et al., 1994; Anigbogu et al., 2000). Phipps et al. (1989) found no differences in the fluxes of lithium and pyridostigmine through human, pig, and rabbit skin *in vitro*. There is, therefore, the need to establish which model closely resembles human skin for both penetration and toxicological studies. Recently, Marro et al. (2001) evaluated the suitability of porcine skin as a model for human skin in iontophoretic studies by comparing the anode-to-cathode and cathode-to-anode delivery of mannitol through both skin types at different pH. They concluded that the isoelectric points 4.4 for pig skin and 4.8 for human skin were close enough and that pig skin showed the same pH-dependent perselectivity for mannitol as did human skin and would, therefore, be an appropriate model for human skin.

12.6 PATHWAYS OF ION TRANSPORT

The predominant pathway for ion transport through the skin remains controversial. Appendages: sweat ducts and hair follicles are thought to be the major pathway for iontophoretic transport through the skin (Grimnes, 1984; Burnette, 1989). This is obviously so in the use of pilocarpine for the diagnosis of cystic fibrosis. Abramson and Gorin (1940) showed that charged dyes delivered iontophoretically produced a dotlike pattern on human skin and the dots were identified as sweat glands. Papa and Kligman (1966) observed a direct link between methylene blue staining of the skin and the location of sweat ducts. Monteiro-Riviere et al. (1994) demonstrated the appendageal pathway for the iontophoretic delivery of mercuric chloride across pig skin *in vivo*. Cullander and Guy (1991) using a vibrating probe electrode identified the largest currents to be in the area of residual hairs. Laser scanning confocal microscopy has been used to elucidate the pathway for the iontophoretic transport of Fe^{2+} and Fe^{3+} ions (Cullander, 1992) as being the sweat glands, hair follicles, and sebaceous glands. Based on these and other studies, the sweat ducts and glands, however, appear to be more important than hair follicles in the transport of ions through the shunts. A schematic of the routes of ion transport across the skin is shown in Figure 12.2.

It is however not correct to assume that all charged transport takes place through the appendages. Walberg (1968) demonstrated that Na^+ and Hg^{2+} could penetrate through guinea pig skin in areas devoid of sweat glands and hair follicles. Millard and Barry (1988) compared the iontophoretic

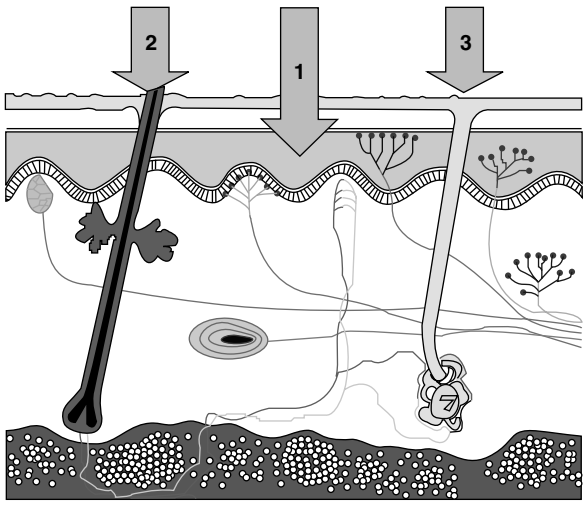


FIGURE 12.2 An illustration showing possible pathways of permanent transport across human skin either through: (1) intact stratum corneum, (2) the hair follicles with interconnected sebaceous glands, or (3) the sweat glands. (From Daniels, R. *Skin Care Forum*, Online Issue 37, 3, 2004. With permission.)

delivery of water and glutamic acid through full thickness human skin and shed snakeskin, which is largely devoid of sweat glands and hair follicles. Iontophoresis was shown to increase the delivery of both materials through snakeskin. Sharata and Burnette (1989) showed that mercuric and nickel ions can diffuse passively between the keratinocytes. Jadoul et al. (1996) concluded from results of fourier transform infrared spectroscopy (FTIR) and small angle x-ray scattering (SAXS) studies on isolated rat and human cadaver skin following prolonged *in vitro* iontophoresis that iontophoresis transport is related to lipid bilayer stacking disorganization.

12.7 FACTORS AFFECTING IONTOPHORETIC DRUG ADMINISTRATION

Several factors come into play when considering iontophoresis for drug delivery. These include the physicochemical properties of the drug in question: the charge, molecular size, and concentration; formulation parameters: choice of vehicle, pH range in which drug is ionic, presence of competing or parasitic ions, viscosity, or mobility (Maurice and Hughes, 1984). Others include physiologic considerations such as appropriate skin site for application; instrumentation, e.g., type of current source, pulsed or constant, and current density. This list is by no means exhaustive but includes some of the more critical factors, which will be considered briefly in this section.

12.7.1 pH

Transdermal iontophoresis achieves the transport of drug molecules into and through the skin under the influence of an applied electric field. This means that the drug candidate should be charged to allow for delivery in therapeutically relevant levels through the skin. The optimum pH for delivery of a drug by iontophoresis is that at which it exists predominantly in the ionic form. This has been demonstrated by Siddiqui et al. (1985, 1989). The pH of peptides, proteins, and other amphoteric substances characterized by their isoelectric point is of particular significance i.e., a pH above which the molecule is anionic and below which it is cationic. For instance, the skin permeability of insulin has been shown to be greater at a pH below its isoelectric point (Siddiqui et al., 1987). Furthermore, the pH gradient encountered in the skin is an important factor in iontophoretic transport. The pH of the skin ranges from 4 to 6 on the outside to about 7.3 in the viable tissues. If, at any time, the drug encounters an environment in which it becomes uncharged, its transport becomes impeded. Thus, for a molecule to be delivered efficiently by iontophoresis, it must remain charged during its transport into and through the skin. For proteins and peptides, iontophoretic transport may be limited to those with isoelectric points below 4 or above 7.3.

12.7.2 Molecular Size

The molecular size of the compound of interest is crucial in predicting the efficiency of its iontophoretic delivery (Srinivasan et al., 1989, 1990; Yoshida and Roberts, 1993). Generally, monovalent positively charged drugs are delivered with greater efficiency by iontophoresis than monovalent negatively charged anions. This has been ascribed to the net negative charge on the skin. The greater the molecular size, the lower the permeability coefficient. Nevertheless, high molecular weight proteins and peptide drugs with molecular weight 3000–5000 Da have been delivered effectively by iontophoresis. As advances in the electronic devices used in iontophoretic drug delivery have evolved, researchers have focused efforts on the delivery of macromolecules ranging from small molecules such as oligonucleotides (Van der Geest et al., 1996; Brand et al., 1998; Davies et al., 2003) to genes (Asahara et al., 1999) and large peptides such as Insulin, MW 3–7 K Da. (Rastogi and Singh, 2002; Kumar et al., 2004).

12.7.3 Concentration

The concentration of the drug in the formulation also affects the flux achieved by iontophoresis. There abound in the literature insurmountable evidence that increasing the concentration of drug in the donor compartment increases proportionately, the flux of the compound e.g., Arginine–Vasopressin (Lelawongs et al., 1989), butyrate (DelTerzo et al., 1989), and diclofenac (Koizumi et al., 1990). A linear relationship between concentration of the drug in the donor solution and flux has been established for gonadotropin releasing hormone (GnRH) and sodium benzoate with flux increasing linearly with increasing concentration (Bellantone et al., 1986). With some drugs, however, increasing the concentration in the donor solution beyond a certain limit appears not to further increase the flux. This was demonstrated for methylphenidate, the steady-state flux of which was found to increase with concentration up to 0.1M (Singh et al., 1997). It was shown recently that increasing the concentration of methotrexate in hydrogels did not further improve the effectiveness of delivery by iontophoresis (Alvarez-Figueroa and Blanco-Méndez, 2001).

12.7.4 Competing Ions

The fraction of current carried by each type of ion in solution is called the transference or transport number. When a migrating ion carries 100% of the current through the membrane, its rate of transport is maximal and its transport number is unity. To control the pH of the donor solution, buffers are often employed. The buffers, however, introduce extraneous ions, which may be of different type but are of the same charge as the drug ion. These are called co-ions and are usually more mobile than the drug ion. The co-ions reduce the fraction of current carried by the drug ion thus resulting in a diminished transdermal flux of the drug. In a recent publication, Mudry et al. (2006) demonstrated the reduction in transport numbers of all cations by addition of co-ions, the magnitude of which was highly dependent on the mobility as well as the molar fraction of the ions. Some workers also employ antioxidants and antimicrobials which

themselves contain co-ions. In addition to these, co-ions can also be introduced from reactions occurring at the electrodes if, for example, platinum is the conducting material. Hydrolysis of water occurs resulting in the generation of hydronium ion at the anode and hydroxyl ion at the cathode. Reducing the amount of competing ions in the drug donor solution will increase the transport efficiency of the drug ions but as there are also endogenous ions in the skin, e.g., sodium, potassium, chloride, bicarbonate, and lactate, which carry an appreciable fraction of the ionic current (Phipps and Gyory, 1992), the transport number of any drug will always be less than unity. Marro et al. (2001b) concluded that the mole fraction of drug relative to competing ions of similar polarity was the determinant of the extent to which it can carry charge across the skin during iontophoresis.

12.7.5 Current

A linear relationship has been established between the iontophoretic fluxes of a number of compounds and the current applied. Examples include lithium (Phipps et al., 1989), thyrotropin releasing hormone (Burnette and Marrero, 1986), mannitol (Burnette and Ongpipattanakul, 1987), gonadotropin releasing hormone (Miller et al., 1990), verapamil (Wearley and Chien, 1989). Tissue distribution of phosphorus following iontophoretic delivery was shown to be proportional to current density (O'Malley and Oester, 1955). This relationship between skin flux and applied current is closely related to fall in skin resistance. As shown in Figure 12.3, the greater the applied current, the lower the steady state skin resistance achieved (Anigbogu et al., 2000). This relationship is, however, seen not to be linear at current densities above 2 mA/cm^2. The rate of transfer of ketoprofen from skin to cutaneous blood in rats was found to be proportional to applied electric current with the enhancement ratios compared to passive delivery being 17 and 73 respectively for 0.14 and 0.70 A/cm^2 (Tashiro et al., 2000). Zhu et al. (2002) reported less skin to skin variability using constant conductance alternating current compared to conventional constant direct current iontophoresis. Plasma and tissue levels of diclofenac sodium in rabbit were found to be proportional to applied current density (Hui et al., 2001) and at up to 0.5 mA/cm^2 of current for six hours in the presence of drug, rabbit skin showed no significant irritation. Overall, therefore, in theory, since the current can be easily modulated, the amount of drug transportable across the skin can be increased by increasing the electric current applied. In practice, however, the limiting factor especially in humans is safety, comfort, and acceptability. The upper limit of current tolerable to humans is thought to be 0.5 mA/cm^2 (Abramson and Gorin, 1941; Ledger, 1992). Increasing the surface area of the electrodes allows for increasing current and, therefore, improving the delivery of some drugs. This is, however, not a linear relationship and may not apply to all drugs (Phipps et al., 1989). In terms of skin barrier properties, it has recently been suggested that the fall in skin impedance following iontophoresis does not necessarily represent damage to the barrier but rather is a response to the relevant electrical potential and ion concentration gradients involved in iontophoresis (Curdy et al., 2002). Kanebako et al. (2002), evaluated the relationship between skin barrier function and direct current applied by measuring short-term resistance. In addition, they demonstrated that the distance between two electrodes affect the barrier function due to the localization of current density in the adjacent electrode. In a follow study (Kanebako et al., 2003), assessed the effect of electrode distance, boundary length, and electrode shape on skin barrier function and percutaneous absorption during iontophoresis. They found that a distance of 2 mm between electrodes decreased skin barrier function and surrounded electrode types were more effective in reducing skin barrier function than paired types.

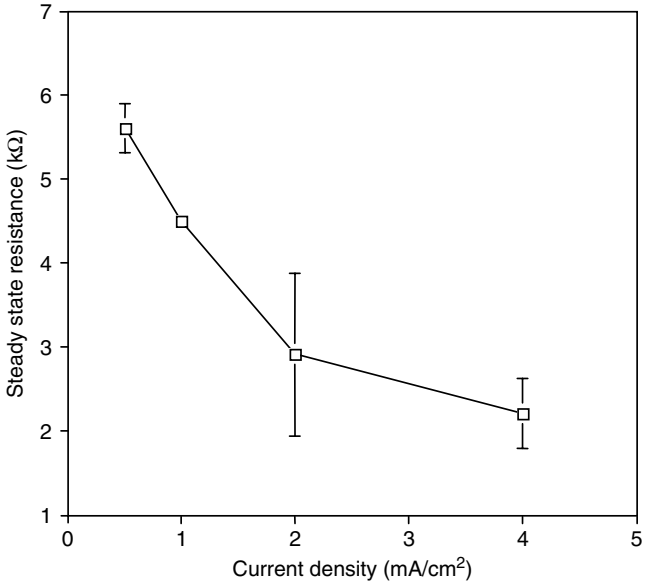


FIGURE 12.3 Apparent steady-state skin resistance as a function of current density. Area of Application = 1cm^2, Duration = 1h, Electrolyte in patch = 0.15M NaCl, pH = 7. (From Anigbogu et al., *Int. J. Pharm*., 200, 203, 2000. With permission.)

12.7.6 Species, Sex, and Site

Iontophoretic deliveries of lithium and pyridostigmine have been found to be comparable in pig, rabbit, and human skin (Phipps et al., 1989). Burnette and Ongpipattanakul (1987) found the iontophoretic fluxes of sodium chloride and mannitol through thigh skin from male and female cadavers to be comparable. Successive iontophoretic delivery of iodine through the same knee in a human volunteer resulted in a constant uptake (Puttemans et al., 1982). Iontophoresis, therefore, decreases the intra- and intersubject variability as well as the influence of site usually observed with passive diffusion. Further studies need to be done, however, to establish the degree to which factors such as race, age, skin thickness, hydration, and status of the skin (healthy or diseased) affect iontophoretic drug delivery. Skin tolerability to electric current appears to be species dependent. Rabbit, which is normally reactive to applied chemicals and is used commonly

in Draize skin irritation test tolerated 1 mA/cm^2 current for up to 1 h without irritation as opposed to human skin for which the upper limit is 0.5 mA/cm^2 (Anigbogu et al., 2000). The same study, however, found skin irritation in rabbit to be tied to applied current density or treatment duration with currents greater than 1 mA/cm^2 applied for periods of 30 min or greater or 1 mA/cm^2 for application times greater than 1 h. In further assessing rabbit skin as a suitable *in vitro* and *in vivo* model for human skin iontophoresis experiments, Nicoli and coworkers (2003) measured the sodium transport number at different pH values for the bathing solution and thus estimated the isoelectric point (IP) of rabbit ear skin to be 2–3. They validated their method by evaluating the transport of mannitol, a model drug through rabbit skin. Applying the same method, they also obtained the same values for human skin, numbers lower than previously reported (Rein, 1924; Marro et al., 2001a). They defined the electroosmotic flow associated with the transport of mannitol at physiologic pH to be in the anode-to-cathode direction. Subsequently, with the same electroosmotic and electrorepulsive contributions to the flux of lidocaine, a model hydrophilic drug observed for human skin and rabbit ear skin, Nicoli et al. concluded that rabbit ear skin is a suitable model for human skin in iontophoretic drug delivery.

Using cellulose membrane as barrier and thus further eliminating inter- and intra-sample variations that could occur with biological membranes, Tiwari and Udupa (2003a) investigated the various parameters affecting iontophoretic transport of ketoralac, a potent nonnarcotic analgesic and anti-inflammatory drug *in vitro*. Their results indicated that increasing drug concentration and current density increased the transport of ketorolac across the membrane while the presence of extraneous ions or increase in the viscosity of the vehicle decreased drug flux and variations in pH from 5.6 to 8 did not influence drug transport. Using glucose as a model nonionizable drug for comparison to evaluate mechanisms of iontophoretic transport of ketorolac, they showed that the total flux of ketorolac resulted from a combination of passive diffusion and electro transport with negligible contribution from electroosmosis. Tiwari and Udupa (2003b) also compared the transdermal transport of ketorolac across full thickness rat skin by iontophoresis and passive diffusion. They investigated electrical, device-related, and the physicochemical factors associated with iontophoretic drug delivery. Iontophoresis resulted in a flux of ketorolac 60 times greater than that achieved by passive diffusion. Increasing the loading dose of drug resulted in a nonlinear increase in the flux of ketorolac. Increasing the ionic strength of the donor solution from 0.06 to 1 M resulted in a sevenfold decrease in the achieved drug flux. Silver–silver and platinum electrodes transported ketorolac with the same efficiency and continuous current was more efficient in the transdermal transport of ketorolac than pulsed current. Pretreatment with ethanol did not increase the flux further by either passive diffusion or iontophoresis i.e., flux by iontophoresis was 60 times greater than for passive transport. Pretreatment with ultrasound followed by either procedure achieved the same flux level as ethanol pretreatment. Pretreatment with D-limonene in ethanol or D-limonene in ethanol and ultrasound significantly enhanced the iontophoretic transport of ketorolac compared to passive diffusion with or without the same pretreatment. Of all the variations in iontophoretic parameters investigated by Tiwari and Udupa, the combined pretreatment of skin with ethanolic D-limonene and ultrasound produced the greatest percutaneous flux of ketorolac.

12.7.7 Continuous versus Pulsed Current

Whether pulsed or continuous direct current should be used, is one of the controversies that exist in the field of iontophoresis. Continuous direct current causes skin polarization with time and this reduces the efficiency of delivery. This can be avoided by using pulsed direct current i.e., direct current delivered periodically. During the "off-period," the skin becomes depolarized returning to near its original state. Chien and co-workers (1989) applying the same current density (0.22 mA/cm^2) over the same 40 min period, were able to deliver twofold level of vasopressin *in vivo* in rabbits using pulsed current from the TPIS described earlier compared to the Phoresor system which delivers constant direct current. They also showed a peak plasma insulin level in 30 min in diabetic rabbits using TPIS (1 mA, 40 min) compared to 1–2 h for the Phoresor system (4 mA, 80 min). Ion transport using pulsed current may, however, be affected by the frequency. If the frequency is high, the efficiency of pulsed delivery is reduced (Bagniefski and Burnette, 1990). While Lui et al. (1988) observed a greater blood glucose reduction in diabetic rats using 2 kHz compared to 1 kHz, Haga et al. (1997) found no significant difference in the decrease in blood glucose levels when frequency was changed from 1 to 2 kHz, in the same species. Pillai et al. (2004) investigated factors affecting the optimization of electronic parameters for the iontophoretic delivery of insulin as a large model peptide. They concluded high flux could be achieved by modulating current density and duration, either low current strength for long duration or high current strength for short duration. Their study further showed that for extended current application, periodic current application was preferable to continuous current application to achieve comparable or higher drug flux. More studies need to be done to explain these discrepancies in results from different studies. Pulsed direct current was shown to be more efficient in delivering two decapeptides, Nafarelin, and LHRH with a greater portion of both drugs intact compared to continuous current (Raiman et al., 2004).

12.7.8 *In vitro–In vivo* Correlation

Recently, we compared the pharmacokinetic and local tissue disposition of diclofenac sodium delivered by iontophoresis and intravenous (IV) infusion (Hui et al., 2001). As shown in Figure 12.4 within 30 min of turning on the current, same plasma concentration was achieved by a 0.2 mA/cm^2 current as the IV infusion. The IV infusion at later time points

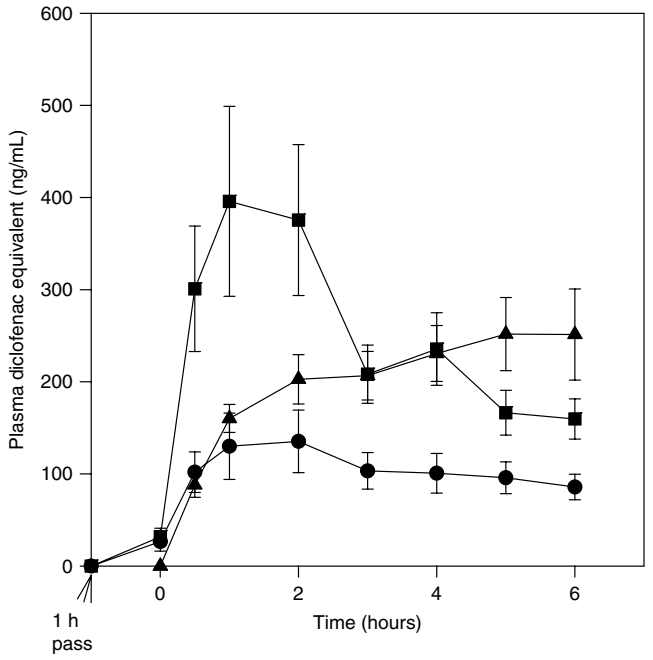


FIGURE 12.4 Plasma Diclofenac sodium concentrations (mean ± SEM, n = 4) over time in rabbit following IV infusion (Dose = 1.25 mg at 0.2 mg/h) and transdermal iontophoresis (Donor concentration =7 mg/mL; pH = 7.4. Key: ▲ IV infusion, ● 0.2 mA/cm^2, ■ 0.5 mA/cm^2. (From Hui et al., *J. Pharm. Sci*., 90, 1272, 2001. With permission.)

produced plasma concentrations surpassing iontophoresis under this conditions at all other time points up to 6 h. Whereas, iontophoresis of diclofenac sodium at 0.5 mA/cm^2 achieved superior plasma concentration than IV infusion from the time the current was initiated till the end of the treatment period. The peak plasma concentration observed between 1–2 h during six hours of iontophoresis was 132 and 371 µg/L with current densities of 0.2 and 0.5 mA/cm^2, respectively. The iontophoretic delivery rates calculated using the C_{max} values from the iontophoresis results and clearance values from the IV infusion data were 0.027 and 0.074 mg/(cm^2/h) for 0.2 and 0.5 mA/cm^2, respectively. The *in vivo* delivery efficiency for diclofenac sodium in rabbit was 0.15 mg/mA.h, a value double that of unpublished data obtained *in vitro* with hairless mouse skin. Bearing in mind differences in experimental conditions and species differences, the *in vitro* and *in vivo* data appear realistic. More studies need to be done in this area.

12.8 ADVANTAGES OF IONTOPHORESIS

Considering the complexity of iontophoresis compared to traditional dosage forms such as tablets, liquids, injections, ointments, and even passive transdermal patches, it must have advantages to enjoy a resurgence of interest.

Transdermal iontophoresis shares many of the advantages of passive transdermal drug delivery including the bypass of hepatic first-pass metabolism, avoidance of gut irritation, controlled drug delivery, and ease of termination of drug-input when necessary. An important consideration is patient compliance. The dosage regimens of many pharmacologic agents available for delivery through other routes pose a challenge to patients. An example is the need to be taken with or without food, dosing frequency (e.g., to be taken every 4–6 h) etc. In addition, the injectable route is particularly uncomfortable to many patients. In fact, Voight et al. (2002) demonstrated that columb-controlled iontophoresis (CCI) was a safe and efficient method of ocular aspirin administration in rabbits and, therefore, could be useful for the treatment of several underlying ocular diseases associated with prostaglandin activity in the eyes such as retinal detachment, diabetic neuropathy and retinopathy of prematurity, scleritis, episleritis, allergic conjunctivitis, and cystoid macular edema without the associated side effects e.g., gastric bleeding, aspirin-induced asthma, hepatotoxicity, platelet dysfunction associated with IV injection. Many drugs, which are available for systemic therapy cannot be delivered through many of the existing traditional dosage forms as they are subject to extensive hepatic first-pass metabolism and variable gut absorption. Many drugs including new biotech drugs (proteins, peptides, and oligonucleosides) (Meyer, 1988; Merino et al., 1997) and local anesthetics such as lidocaine (Gangarosa, 1981), which would have to be injected to derive maximum benefit, have been delivered efficiently using iontophoresis.

Since the rate of drug delivery is generally proportional to the applied current, the rate of input can, therefore, be preprogramed on an individual basis (Banga and Chien, 1988). The controllability of the device would eliminate the peaks and troughs in blood levels seen with oral dosing and injections. Patients can titrate their intake of drugs as required.

12.9 PROBLEMS ASSOCIATED WITH IONTOPHORESIS

Only a fraction of the charge introduced in iontophoresis is delivered suggesting that iontophoresis is not necessarily as efficient as theoretically proposed (Sage and Riviere, 1992).

Of more serious consideration, however, are the unwanted skin effects of iontophoresis arising from the system itself or drug formulation. Typically, side effects of iontophoresis with low voltage electrodes properly used are minimal; nevertheless, must be considered. These include itching, erythema, edema, small punctate lesions, and sometimes burns. A slight feeling of warmth and tingling is generally associated with iontophoresis (Kellog et al., 1989; Zeltzer et al., 1991; Ledger, 1992; Maloney et al., 1992). Erythema is also commonly reported and is thought to arise from skin polarization associated with continuous direct current. To minimize this, pulsed direct current has been advocated. Electric shock can occur when high current density is directed at the skin. To minimize this, the current should be increased slowly from zero to the maximum desired current level acceptable to the patient. Similarly, at the end of the procedure, current should

be returned from the maximum to zero in a stepwise manner. The effect of current on nerve fibers is thought to be responsible for the itching, tingling, and erythema.

The histological and functional changes that occur in animal skin following iontophoresis have been studied. Under similar delivery conditions (i.e., drug concentration, current density, and duration) as are used in humans, Moteiro-Riviere (1990) studied structural changes in porcine skin following iontophoresis of lidocaine. Light microscopy revealed epidermal changes. He, however, noted that similar changes were not observed following iontophoresis of other compounds suggesting the effects were largely due to the lidocaine rather than the electric current. Cho and Kitamura (1988) iontophoresing lidocaine through the tympanic membrane of the guinea pig, observed a loss of adhesion of the epidermis to underlying connective tissue and retraction of noncornified epidermal cells. Jadoul et al. (1996) used FTIR and SAXS to study isolated rat skin and human skin from cadaver following prolonged iontophoresis. While FTIR revealed transient increases in the hydration of the outer layers of the stratum corneum but no increase in lipid fluidity, SAXS showed that iontophoresis induced a disorganization of the lipid layers. This was also reversible within days of the procedure. Using wide-angle x-ray scattering (WAXS), the authors did not find evidence either of modification of the intralamellar crystalline packing of lipids or of keratin.

The answer to what should be the upper limit of current tolerable to humans is not very straight forward, as what may be just discernible to one patient may be uncomfortable to another. Generally, however, 0.5 mA/cm^2 is cited (e.g., Abramson and Gorin, 1941; Banga and Chien, 1988; Ledger, 1992). Molitor and Fernandez (1939) found that the greater the surface area of the electrode, the larger the tolerable current but the relationship is curvilinear. Small punctate lesions are associated with electric current traveling through a path of least resistance into the skin. Common sense thus dictates that iontophoresis should not be used on skin showing signs of damage. Pain and burns arising from iontophoresis are linked to electrochemical reactions, which occur at the electrodes and involve the electrolysis of water to generate hydronium and hydroxyl ions resulting in pH changes (Sanderson et al., 1989). Much earlier, however, Molitor and Fernandez (1939) using continuous flow electrodes, which did not generate hydroxyl and hydronium ions and, therefore, did not produce any pH changes, showed that burns could not solely be related to pH changes.

Erythema is the most common side effect associated with iontophoresis and could be due to nonspecific skin irritation such as occur with the delivery of an irritant drug. Erythema may be due to a direct effect of electric current on blood vessels or current induced release of histamine, prostaglandins, or other neurotransmitters leading to local vasodilatation of the affected area. It has also been suggested that electric current can stimulate specific classes of noiceptors, the C-fibers causing them to release the potent vasodilators, substance P, and calcitonin gene-related peptide (CGRP) (Brain and Edwardson, 1989; Dalsgaard et al., 1989). Whatever the cause of the erythema, it is usually transient and not associated with any permanent changes in the skin.

Delayed-type contact sensitivity to components of the iontophoresis system, electrodes, and electrode gels (Fisher, 1978; Zugerman, 1982; Schwartz and Clendenning, 1988), or to the drug being delivered (Teyssandier et al., 1977; Holdiness, 1989) has been reported.

Another consideration in choosing iontophoresis for drug delivery is cost. Iontophoresis requires a power source to supply electrical energy. Even though the power requirement for a unit delivery may be small, repeated applications would require a considerable investment in battery supply. Better batteries than those currently used need to be developed.

An important consideration in the use of iontophoresis for drug delivery especially for unstable compounds is whether they are delivered intact or degraded. This has recently been addressed by Brand et al. (2001) who delivered antisense, phosphorothioate oligonucleotides into rats by iontophoresis. They were able to measure the decline in CYP3A2 levels suggesting that the antisense agent was successfully delivered in sufficient therapeutic amounts and intact. Raiman et al. (2004) compared the effect of constant and pulsed iontophoresis on the delivery and stability of LHRH and Nafarelin in human skin. They observed that pulsed direct current was more efficient in the transport of both compounds across human epidermis compared to constant current with the percentage of intact LHRH delivered being slightly higher with pulsed current with five degradation products detected for either method. With Nafarelin, however, the peptide was delivered completely intact using pulsed current with no degradation products detected in the receiver medium. A more stable analog of LHRH acting as a superagonist has also been used in place of hormone to ensure that clinically relevant amounts of the drug is delivered intact across full thickness hairless mouse skin (Miller et al., 1990).

Even though proteolytic activity is considerably less in the skin compared to the oral mucosa, biotransformation of drugs nevertheless, occurs in the skin and can be an important issue to be considered in iontophoresis. Enzymatic degradation of proteins and peptides during iontophoresis has indeed been reported (Steinstrasser and Meckle, 1995). The coapplication of protease inhibitors is one way of reducing enzymatic degradation of peptides and has been addressed as illustrated in the successful *in vivo* nasal delivery of vasopressin in rats and salmon calcitonin across rat skin *in vivo* (Morimoto et al., 1991, 1992).

Iontophoresis is contraindicated in patients with high susceptibility to applied currents and in patients with known hypersensitivity to the drug in question. Iontophoresis should be avoided in patients with electrically sensitive implants such as pace makers. To improve acceptability by both prescribers and patients, more studies need to be done in the field of iontophoresis to minimize unwanted side effects and improve safety.

12.10 DIFFERENTIAL CLINICAL DIAGNOSES, CURRENT AND FUTURE TREATMENT MODALITIES USING IONTOPHORESIS

Lim et al. (2004) compared the response of normal subjects and patients with chronic heart failure (CHF) to iontophoresed Urotensin II (U-II), a vasoactive peptide. They observed that while normal subjects exhibited a dose-related skin microvasculature vasodilator response to U-II measured by the laser Doppler velocimeter, in contrast, patients with CHF exhibited a dose-related vasoconstrictor response. They, therefore, concluded that in addition to the direct effects of U-II on the myocardium, it may contribute to the increased peripheral vascular tone characteristic of CHF and therefore, its blockade may be a target in the treatment of CHF.

Because injection of local anesthetics at sites of dermatologic procedures in children is not viewed positively due to the pain and fear associated with injections, iontophoresis has been explored as a viable option by different groups of investigators. A few of the studies and the findings are detailed here. In a randomized, cross over study, Galinkin et al. (2002) compared local anesthesia achieved by lidocaine iontophoresis and a eutectic mixture of local anesthetics (EMLA®) in 26 patients, ages 2–16 years requiring multiple venous cannulation. During the third session, each patient received his or her preferred method of anesthesia, and pain during venipuncture was assessed by the patients, parents, investigator, and technician performing the blood draw using a 100 mm visual analog scale (VAS). The observers also used the Eastern Ontario pain scale to rate the children's pain. They concluded that both methods, iontophoresis of lidocaine or EMLA provided the same level of pain relief for insertion of IV catheters and, therefore, that lidocaine iontophoresis would be a useful noninvasive method of achieving dermal anesthesia for venous cannulation.

Zempsky and Parkinson (2003) performed a prospective, double blind placebo controlled assessment of iontophoresis of 2% lidocaine with 1:100,000 epinephrine in 60 children, ages 4–16 years requiring dermatologic procedures. Of the 31 pediatric patients that received lidocaine, only two required supplemental anesthesia compared to 27 of the patients who received placebo ($p < 0.001$). The lidocaine treated patients reported a significantly lower pain on the Oucher pain scale following their procedure ($p < 0.001$). The parents and investigators also rated the pain lower ($p < 0.001$). Erythema and blanching that was observed on the treated sites in 58 of the 60 patients resolved within 1 h post treatment. Using the Northstar ionotophoretic drug delivery system (IDDS), Kearns et al. (2003) delivered Lidocaine in children, 5–10 years using a current of 1.78 mA. Local anesthesia was achieved in 10 min compared to 40–60 min normally achieved by eutectic mixtures of anesthetics and EMLA cream. In addition, no skin irritation was observed for up to 24 h following iontophoresis and no subject reported any pain or discomfort with this method of lidocaine delivery. With minimal systemic bioavailability of lidocaine in all subjects (10 ng/mL) and excellent tolerability, they concluded that IDDS was suitable for clinical use in pediatrics. The conclusion from these studies is that iontophoresing lidocaine is a safe, well-tolerated method of topical anesthesia in pediatric patients before dermatologic procedures.

With the obvious potential advantages of ocular iontophoresis over traditional ocular drug delivery methods e.g., drops and ointments including the ability to rapidly deliver a broad range of drugs including large peptides and oligonucleotides beyond the anterior segment of the eye, Parkinson and co-workers (2003) investigated the tolerance of ocular iontophoresis in healthy volunteers. Using Ocuphor™ hydrogel drug delivery applicators, they transclerally iontorephesed isotonic balanced solution in 24 male and female subjects, in a three-period crossover study in which 16 subjects received 0 mA and 2 DC currents from a choice of 0.1, 0.5, 1, 2, 3, 4 mA for 20 min. Six subjects received either 3 mA for 20 min or 1.5 mA for 40 min (equivalent to 60 mAmin total charge). Using a subjective VAS and an objective ophthalmic assessment before and up to 22 h following treatment, to evaluate safety and tolerance of the treatment parameters they found a good tolerance and no clinically significant ophthalmic changes occurring with 0–3 mA current delivery for 20 min or 1.5 mA current application for 40 min. Two of the four subjects exposed to 4 mA current for 20 min reported a burning sensation under the applicators, which resolved by 22 h postdosing.

Eljarrat-Binstock and colleagues (2004) performed ocular delivery of gentamycin by iontophoresis, from a drug-loaded disposable hydroxyethyl methacrylate (HEMA) hydrogel, with an applied current of 1 mA for 1 min in rabbit eye. The study used three control groups consisting of animals with mock iontophoretic treatment for 1 min i.e., no current applied, animals given subconjunctival injection of 0.25 mL of 40 mg/mL gentamycin solution, and animals treated with topical eye drops of fortified gentamycin (1.4%) applied every 5 min for 1 h. They measured gentamycin levels in the cornea and aqueous humor by fluorescence polarization immunoassay. Peak concentrations of gentamycin in the cornea and aqueous humor were achieved at 0 and 2 h respectively following iontophoresis. The levels of gentamycin following a single iontophoretic treatment were 12–15 times higher than from injection or topical instillation and were much higher still for mock iontophoresis. Therapeutic concentrations of gentamycin were maintained for 8 h following cessation of current. This suggests that short duration iontophoresis has potential application for increasing the penetration of antibiotics and other drugs to the anterior portions of the eye and maintaining therapeutic levels of drug at the desired target for long periods of time. Further work done by Raiskup et al. (2006) using a solid hydrogel made from HEMA cross-linked with ethyleneglycol dimethacrylate (EGDMA) as a probe to deliver gentamycin into rabbit by iontophoresis by applying 1.5 mA current for 60 s compared to eye drops with 1.4% gentamycin, observed high drug concentrations in the sclera and in the retina and in the sclera four hours after transscleral

iontophoresis, with the lowest concentration observed in vitreous fluid. They concluded that HEMA/EGDMA hydrogels are promising for more efficient ocular iontophoresis of gentamycin.

Transcleral methylprednisolone (solumedrol) iontophoresis was used successfully to treat severe acute corneal graft rejection (Halhal et al., 2003). Treatment consisted of solumedrol iontophoresis under topical anesthesia using a current of 1.5 mA for 3 min, once a day for 3 days. Topical corticoid therapy using dexamethasone was reduced to three drops per day. Ocular iontophoresis was well tolerated with no patient complaints and no observed side effects. The therapeutic efficacy of solumedrol iontophoresis in corneal rejection was assessed by the achievement of corneal transparency, visual acuity, and measurement of corneal inflammation parameters, which had all cleared.

Intraocular retinoblastoma is treated by systemic chemotherapeutic delivery of carboplastin. In addition to significant morbidity and mortality associated with systemic carboplastin, it may be associated with future occurrences of cancer in pediatric patients because of mutation at the RB-1 gene. Hayden et al. (2004) explored coulomb-controlled ocular iontophoretic delivery of carboplastin (5 mA/cm^2 for 20 min) as an alternative and safer direct delivery of the drug using a single IV infusion of carboplatin and a single subjunctival injection of carboplastin in New Zealand white rabbits as controls. Analyses of the retina, choroids, vitrous humor, and optic nerve showed that iontophoresis resulted in significantly higher levels of carboplastin compared to both IV injection and subconjunctival injection of the drug. In addition, much higher systemic plasma concentrations of carboplastin were achieved following IV injection than by subconjunctival injection and coulomb-controlled iontophoresis with no ocular toxicity observed following the later methods of delivery. They thus, concluded that iontophoresis might offer a means of delivering high levels of the cytotoxic drug directly to target tissues for the clinical treatment of retinoblastoma with concomitant reduction in the systemic exposure.

Khan et al. (2003) iontophoresed Acetylcholine (ACH) and sodium nitroprusside (SNP), in 145 normal healthy children aged 11–14 years and measured skin microvasular responses by laser Doppler imaging. They divided the subjects into a quintile, based on 2-h postfeeding glucose levels. The subjects in the upper glucose quintile expressed significantly lower vasodilation to ACH and SNP than those in the lower quintile. In some of the children, the macrovascular function was negatively associated with abnormal adiposity. In addition, they found that waist to hip ratio and fasting insulin resistance was higher in the upper quintile than the lower quintile. Fasting triglyceride were also greater in the upper quintile than the lower. Their overall conclusion is that risk factors for adult cardiovascular disease begin to cluster much earlier in life in normal children and these may have important consequences for the risk of developing artherosclerosis later in life. Thus, screening much earlier in life by iontophoresing ACH and SNP and measuring microvascular response, and taking preventative actions in otherwise normal looking children will bode well for their health as adults.

In a separate study, using iontophoresis, Koïtka and coworkers (2004) explored the causative mechanism for the prevalence of foot ulcers in diabetics. They iontophoresed two agonists, ACH and SNP and compared the cutaneous blood flow in normal and diabetic patients using Laser Doppler flowmetry and found that while normal subjects had pressure induced vasodilation at the foot level, it was largely absent in diabetic patients. They postulated that the absence of cutaneous vasodilator response to progressive pressure strain might be related to interactions between changes in unmyelinated C fibers and the endothelium and, therefore, relevant in the high prevalence of foot ulcers in diabetics.

Iodide was applied in two groups of patients suffering from dry eye syndrome or keracojunctivitis sicca, 16 of them by iontophoresis and 12 patients without current for a total of 10 days (Howarth-Winter et al., 2005). The study used 0.5% sodium iodide solution of pH 8 at which the iodide ions were negatively charged. Since the treatment was over an extended period of time, for safety purposes, a lower current, 0.2 mA applied for 7 min. Based on the significantly greater improvement in clinical symptoms and reduced frequency of use of artificial tears observed in the patients that received iontophoresed iodide, versus the noniontophoresed group, the researchers concluded that iontophoresis was a safe and effective method of dry eye syndrome. Leboulanger et al. (2004) evaluated reverse iontophoresis as a noninvasive alternative method of monitoring lithium ion *in vivo* in bipolar and schizo-affective disorder patients in place of blood measurements. They extracted lithium and other cations (sodium, potassium, and calcium) from 23 bipolar and schizo-affective patients over a 2-h period with an applied current of 0.8 mA and accurately assayed all cations by ion chromatography with blood samples drawn from the same patients serving as controls. Normalization of the lithium flux with that of sodium, which served as an internal standard allowed them to correctly calibrate the method with the amount of lithium ion extracted being proportional to the serum concentration. Nicoli and Santi (2006) were able to measure Amikacin, a model aminoglycoside antibiotic with broad spectrum Gram-negative activity, delivered by anodal iontophoresis at the cathode by reverse iontophoresis, suggesting that if fully developed and optimized, this might offer an accurate means of monitoring the antibiotic topically applied.

Sakamoto et al. (2004) topically iontophoresed antisense oligonucleotide for mouse interleukin [IL-10 (AS6)] onto lesions on mouse skin with established dermatitis of a human atopic dermatitis model (NC/Nga mice). 30% of the applied dose of AS6 penetrated the skin and was distributed in the epidermis and upper dermis resulting in decreased levels of mRNA and protein of IL-10 in the lesions of NC/Nga mice, with IL-4 levels unaffected. Repeated applications of AS6 inhibited IL-10 production and resolved the skin lesions. Though the precise mechanism(s) by which inhibition of IL-10 resolves skin lesions is unknown, the study demonstrated that this may facilitate the

treatment of atopic dermatitis in human subjects and further contribute to the knowledge of the disease etiology.

12.11 APPLICATIONS OF IONTOPHORESIS IN DERMATOLOGY

In the past, iontophoresis was found useful in local delivery of pharmacologic agents. Iontophoresis has been used for the treatment of various dermatologic conditions including lupus vulgaris using zinc. Before the advent of antibiotics, infections were treated by the iontophoresis of metals e.g., the treatment of streptococcal infections with copper sulfate. Other conditions that have benefited from the use of iontophoresis include lichen planus, scleroderma, plantar warts, hyperhydrosis, infected burn wounds, and achieving local anesthesia. Even though Botulinum toxin type A has recently been added to the treatment algorithm for axillary or palmoplantar hyperhydrosis (Lowe et al., 2002; Lauchli and Burg, 2003), because of the pain and cost associated with the treatment, iontophoresis with its long established safety history, remains an inexpensive and popular treatment choice especially for palmoplantar hyperhydrosis (Karacoc et al., 2002; Thomas et al., 2004; Haider and Solish, 2005). Bursitis and other musculoskeletal conditions have been treated with iontophoresed corticoids (Harris, 1982). Summaries of dermatologic applications of iontophoresis have been made by Sloan and Soltani (1986) and Singh and Maibach (1994), Rai and Srinivas (2005). Of greater interest in this era is the use of iontophoresis for controlled systemic drug delivery and for targeting deep tissue penetration. Recently, "reverse iontophoresis" involving the extraction of material from the body for the purposes of clinical chemistry has been described (Guy, 1995; Guy et al., 1996). Although glucose is not charged, iontophoresis can markedly increase its passage across the skin by electroosmosis (Merino et al., 1997; Tierney et al., 2000) and this has been applied for the noninvasive monitoring of diabetics' blood sugar levels (Tamada et al., 1995; Svedman and Svedman, 1997; Tierney et al., 2001a,b; Potts et al., 2002). In addition to drug delivery, with the availability of sensitive assay methods, iontophoresis is thus being touted as a diagnostic tool. The "Glucowatch® Biographer" was in fact approved by the Food and Drug Administration in 2001 and launched in the United States in April 2002. Sieg et al. (2004a,b) explored the concept of calibration-free glucose monitoring using sodium ion as an internal standard with aims of improving the reverse iontophoresis method of assessing glycemia in diabetics employed in the Glucowatch technology. They found that the glucose ions extracted iontophoretically, reflected the blood glucose concentration profiles and that sodium extraction was essentially constant mirroring the known constant systemic concentration of sodium in contrast to the variation in extracted potassium ions observed in about two-thirds of the study population. They concluded that calibration with a blood sample was not necessary in using sodium ion as an internal standard in blood glucose monitoring. Recurrence of cold sores has been attributed to difficulties in getting sufficient drug amounts to the infected tissues. Morrel et al. (2006) performed a multicenter placebo controlled clinical trial in which they explored a low voltage iontophoretic delivery of 5% acyclovir as well as topical application in 200 patients with an incipient cold sore outbreak at the erythema or papular/edema lesion stage. The results demonstrated that the outbreak was aborted at a significantly quicker pace in subjects that received active iontophoresis than the vehicle group. The investigators suggested that iontophoresis should be further investigated for the treatment of herpes labialis.

12.12 DEVICES APPROVED OR AWAITING APPROVAL

Iomed's Iontocaine (Numby stuff®), a mixture of 2% lidocaine HCl and epinephrine 1:100,000 (0.01 mg/mL) in the phoresor iontophoresis system was approved in 1995 and is indicated for local dermal anesthesia during local surgeries. Empi Dupel® electrodes approved as a device by the FDA and not in association with any active, has been used since 1992 by sports medicine practitioners for localized tissue delivery of dexamethasone and other corticosteroids for the relief of acute local inflammatory and other sports injury related conditions. With the approval of the Glucowatch Biographer, it is more likely in the future that iontophoresis will be used as a diagnostic tool for other disease conditions. Vyteris is awaiting approval for an iontophoresis device to deliver lidocaine for anesthesia in children (Cleary, 2003). The Vyteris device has the advantage of being prefilled with drug and is current controlled, thus reducing irritation and inducing a rapid onset of action, achieving local anesthesia in 10 min (Rhodes, 2002). ALZA Corporation, a division of Johnson & Johnson received marketing authorization from the European commission to market its IONSYS™ in the European Union (PR Newswire, 2006). This is the first compact (weight: 15 g, dimensions, $3.3'' \times 1.9''$), noninvasive patient-activated transdermal system (PATS) delivering 40 µg/dose of fentanyl hydrochloride, a narcotic analgesic over a 10 min period for use in patients with acute postoperative pain. Patients can initiate for up to 6 doses/h for up to 24 h from the time of first dosing or a maximum of 80 doses, whichever comes first (Koo, 2005). Scale up and launch of the product is expected in 2007. ALZA having originally submitted an NDA for licensure of IONSYS in the United States to the FDA in September 2003 and receiving an approvable letter in mid 2004 had since resubmitted an NDA for the device in November 2005 (Medical Letter, 2006) and received approval for marketing the device on May 23, 2006 (Reuters, 2006).

12.13 CONCLUSIONS

Strides in the biotech industry will continue to result in a large number of drugs, many of which would be proteins,

peptides, and oligonucleotides, most of which at present can only be delivered by the injectable route. In addition, many of the old drugs already in use have the same dosage form requirements with its associated problems. Iontophoresis provides an attractive alternative to the existing dosage forms in delivering these drugs both for local as well as systemic indications. The fact that it could allow for a programmable rate-controlled delivery of drugs makes it particularly attractive. Considerable progress has been made in hydrogel technology and the world of formulation science as well as in microelectronics and battery technology, all of which have been harnessed in improving iontophoresis technology. With high throughput low-cost manufacturing capability, many controllable, miniaturized, iontophoretic devices have been fabricated and are available for the iontophoresis delivery of many suitable drug candidates. The recent approval of IONSYS for iontophoretic delivery of fentanyl by both EU- and US regulatory bodies, will more than likely bolster the development efforts being made by many companies in this area. It is not far reaching to predict that the medical world should expect many more marketing approvals for iontophoretic delivery systems for drugs of serious therapeutic consequences in the not too distant future.

Like any new technology, research will continue to better define and fine-tune the parameters to better maximize the safety, acceptability, and efficiency of iontophoresis as a routine dosage form.

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13 Irritant Dermatitis: Clinical Heterogeneity and Contributing Factors

Sara Weltfriend and Howard I. Maibach

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13.1 CLINICAL ASPECTS

Irritant contact dermatitis occurs when chemicals or physical agents damage the surface of the skin. The clinical presentation is highly variable and depends on many factors including amount and strength of the irritant, length and frequency of exposure, environmental factors, and skin susceptibility. In addition to the typical clinical features of dermatitis, the clinical presentations may change according to the physicochemical nature of the irritant concerned (Table 13.1). Ulcerative lesions can develop from skin contact with strong acids or strong alkalis like calcium oxide, calcium hydroxide, sodium hydroxide, sodium metasilicate, sodium silicate, potassium cyanide, and trisodium phosphate. Compounds of beryllium, arsenic, or cadmium are also capable of inducing ulcerations. Chrome ulcers are the most common type of cutaneous ulcers induced by irritants. Solvents such as acrylonitrile and carbon bisulfide as well as gaseous ethylene oxide are examples of contactants that may induce ulceration in certain occupations. Cutaneous ulcers

TABLE 13.1
Clinical Features That May Suggest the Etiology of Irritant Contact Dermatitis

Ulcerations	Strong acids, especially chromic, hydrofluoric, nitric, hydrochloric, sulfuric. Strong alkalis, especially calcium oxide, sodium hydroxide, potassium hydroxide, ammonium hydroxide, calcium hydroxide, sodium metasilicate, sodium silicate, potassium cyanide, trisodium phosphate. Salts, especially arsenic trioxide, dichromates. Solvents, especially acrylonitrile, carbon bisulfide. Gases, especially ethylene oxide, acrylonitrile
Folliculitis and acneiform	Arsenic trioxide, glass fibers, oils and greases, tar, asphalt. Chlorinated naphthalenes, polyhalogenated biphenyls, and others.
Miliaria	Occlusive clothing and dressing, adhesive tape, ultraviolet, infrared, aluminum chloride
Pigmentary alterations	Hyperpigmentation, any irritant or allergen, especially phototoxic agents such as psoralens, tar, asphalt, phototoxic plants, and others. Metals, such as inorganic arsenic (systemically), silver, gold, bismuth, mercury. Radiation, ultraviolet, infrared, microwave, ionizing. Hypopigmentation, *p-tert*-amylphenol, *p-tert*-butylphenol, hydroquinone, monobenzyl ethyl hydroquinone, monomethyl hydroquinone ether, *p-tert*-catechol, *p*-cresol, 3-hydroxyanisole, butylated hydroxyanisole, 1-*tert*-butyl-3, 4-catechol, 1-isopropyl-3, 4-catechol, 4-hydroxypropriophenone
Alopecia	Borax, chloroprene dimers
Urticaria	Numerous chemicals, cosmetics, animal products, foods, plants, textile, woods
Granulomas	Keratin, silica, beryllium, talc, cotton fibers, bacteria, fungi, parasites, and parasite parts

develop from the direct corrosive and necrotizing effect of the chemical on the living tissue. Exposed areas, where both friction and chemical irritation are associated, are most susceptible for ulcers; minor preceding trauma in the exposed skin increases the risk. The ulcers tend to be deeper, with an undermined thickened border, and the exudate under the covering crusts predisposes to infection. Cutaneous granulomas are considered a variant of irritant contact dermatitis, caused by a biologically inactive substance inoculated into the skin, where macrophages respond with phagocytosis to the foreign body inoculation, and even giant cells may be seen (Epstein, 1983). A granuloma appears as a focal, tumid lesion persisting chronically in its primary site. It is subjectively symptomless. Powders, lead, and metals such as metallic mercury, beryllium, and silica are examples of substances that elicit toxic skin granulomas (Kresbach et al., 1971).

The actual types of irritant contact dermatitis, with reference to major characteristics in the clinical appearance, are listed in Table 13.2.

TABLE 13.2
Clinical Classification of Irritation

Irritation	Onset	Prognosis
Acute (primary) irritant dermatitis	Acute, often single exposure	Good
Irritant reaction	Acute, often multiple exposures	Good
Delayed acute irritant dermatitis	Delayed, 12–24 h or longer	Good
Subjective irritation	Acute	Excellent
Suberythematous irritation	Slowly developing	Variable
Cumulative irritant contact dermatitis	Slowly developing (weeks to months)	Variable
Traumiterative dermatitis	Slowly developing (weeks to months)	variable
Traumatic irritant dermatitis	Slowly developing following trauma	Variable
Pustular and acneiform dermatitis	Moderate-slow developing (weeks to months)	Variable
Exsiccation eczematoid	Moderate-slow developing (weeks to months)	Variable
Friction dermatitis	Moderate-slow developing (weeks to months)	Variable

13.1.1 Acute Irritant Dermatitis (Primary Irritation)

When exposure is sufficient and the offending agent is potent, such as acids or alkaline solutions, classic symptoms of acute skin irritation are seen. Contact with a strong primary irritant is often accidental, and an acute irritant dermatitis is elicited in almost anyone independent of constitutional susceptibility. This classic, acutely developing dermatitis usually heals soon after exposure. The healing of acute irritant dermatitis is described as a *decrescendo phenomenon,* where the irritant reaction quickly peaks and then immediately begins to heal upon removal of irritant. In unusual cases the dermatitis may persist for months after exposure, followed by complete resolution. The availability of the material Safety Data Sheet and data from the single-application Draize rabbit test combined with activities of industrial hygienists and other informed personnel greatly decreased the frequency of such dermatitis in industry. Further educational efforts and appropriate industrial engineering should make this form of irritation a rarity.

13.1.2 Delayed, Acute Irritant Contact Dermatitis

Some chemicals like anthralin (dithranol), benzalkonium chloride, and hydrofluoric acid are chemicals, which may elicit a retarded inflammatory response, so that inflammation is not seen until 8–24 h or more after exposure (Malten et al., 1979; Lovell et al., 1985) (Table 13.3). Except for the delayed onset, the clinical appearance and course resemble those of acute irritant contact dermatitis. The delayed acute irritant

TABLE 13.3
Chemicals Inducing Delayed Acute Irritation

Anthralin
Bis(2-chloroethyl)sulfide
Butanedioldiacrylate
Dichloro(2-chlorovinyl)arsine
Epichlorhydrin
Ethylene oxide
Hydrofluoric acid
Hexanedioldiacrylate
Hydroxypropylacrylate
Podophyllin
Propane sulfone

dermatitis, because of its delayed onset, is often confused with allergic contact dermatitis; appropriately performed diagnostic patch tests easily separate the two.

13.1.3 Irritant Reaction

Individuals extensively exposed to irritants, in the first months of exposure, often develop erythematous, chapped skin on the dorsum of the hands and fingers. This irritant reaction (Fregert, 1981; Griffiths and Wilkinson, 1985; Hjorth and Avnstorp, 1986) may be considered a preeczematous expression of acute skin irritation. It is frequently seen in hairdressers and variable wet work-performing employees repeatedly exposed. Repeated irritant reactions sometimes lead to contact dermatitis, with good prognosis, although chronic contact dermatitis may also develop.

13.1.4 Subjective/Sensory Irritation

Subjective irritation is experienced by some individuals ("stingers") in contact with certain chemicals (Frosch and Kligman, 1982; Lammintausta et al., 1988b; Jourdain et al., 2005). Itching, stinging, or tingling is experienced, for example, from skin contact with lactic acid, which is a model for no visible cutaneous irritation. The threshold for this reaction varies between subjects, independent of susceptibility to other irritation types. The quality as well as the concentration of the exposing agent is also important, and neural pathways may be contributory, but the pathomechanism is unknown. Some sensory irritation may be subclinical contact urticaria. Screening raw ingredients and final formulations in the guinea pig ear swelling test (Lahti and Maibach, 1985) or the human forehead assay allows us to minimize the amount of subclinical contact urticaria. Although subjective irritation may have a neural component, some studies suggest an altered baseline biophysical parameters representing a trend toward barrier impairment (Seidenari et al., 1998). Additionally, the blood vessel may be more responsive in "stingers" than in nonstingers (Lammintausta et al., 1988b; Berardesca et al., 1991a). At least 10% of women complain of stinging with certain facial products; thus, further work is needed to develop a strategy to overcome this type of discomfort.

13.1.5 Suberythematous Irritation

In the early stages of skin irritation, subtle skin damage may occur without visible inflammation. As a correlate of no visible irritation, objectively registered alterations in the damaged epidermis have been reported (van der Valk et al., 1985; Lammintausta et al., 1988b; Charbonnier et al., 2001). Common symptoms of suberythematous irritation include burning, itching, or stinging. Consumer dissatisfaction with many chemicals may result from exposure to this low-grade irritation; thus, the patient feels more than the physician observes. It is customary in Japan to screen new chemicals, cosmetics, and textiles for subtle signs of stratum corneum damage, employing replicas of stratum corneum (the Kawai method) (Kawai, 1971).

13.1.6 Cumulative Irritant Dermatitis

Multiple subthreshold skin insults induced by repeated applications of weak irritants may lead to cumulative cutaneous irritation. In cumulative cutaneous irritation, the frequency of exposure is too high in relation to the skin recovery time. Acute irritant skin reaction is not seen in the majority of patients, but mild or moderate invisible skin changes. Repeated skin exposures and minor reactions lead to a manifest dermatitis when the irritant load exceeds the threshold for visible effects. The development of a cumulative irritant dermatitis was carefully documented by Malten and den Arend (1978) and Malten et al. (1979). Classic signs are erythema and increasing dryness, followed by hyperkeratosis with frequent cracking and occasional erythema. Cumulative irritant dermatitis is the most common type of irritant contact dermatitis. This syndrome may develop after days, weeks, or years of subtle exposure to chemical substances. Variation in individual susceptibility increases the multiplicity of clinical findings. Delayed onset and variable attack lead to confusion with allergic contact dermatitis. To rule out allergic etiology, appropriate diagnostic patch testing is indicated. Models of cumulative irritant dermatitis have been developed (Freeman and Maibach, 1988; Widmer et al., 1994).

13.1.7 Traumiterative Irritant Dermatitis

Traumiterative irritant dermatitis, in the older German literature ("traumiterative" = traumas repeating) (von Hagerman, 1957; Agrup, 1969), is a consequence of too frequent repetition of one impairing factor. This syndrome and cumulative irritant dermatitis are very similar clinically.

13.1.8 Traumatic Irritant Dermatitis

Traumatic irritant dermatitis develops after acute skin trauma. The skin does not completely heal, but erythema, vesicles or vesicopapules, and scaling appear. The clinical course later

resembles nummular (coin-shaped) dermatitis. This may occur after burns or lacerations and after acute irritant dermatitis: It may be compounded by a concurrent allergen exposure. The healing period is generally prolonged. Often these patients are considered to have a factitial dermatitis because of a healing phase followed by exacerbation. Although factitial (unnatural) aspects may occur in some patients, this peculiar form of irritation appears to be a disease *sui generis*. Its chronicity and recalcitrance to therapy provides a challenge to both patient and physician. We have no information explaining why the occasional patient develops this phenomenon, and how this patient differs from the general population. Many such patients are considered factitial in origin if the dermatologist is unaware of the syndrome.

13.1.9 Pustular and Acneiform Irritant Dermatitis

Pustular and acneiform irritant dermatitis may develop from exposure to metals, oils and greases, tar, asphalt, chlorinated naphthalene, and polyhalogenated naphthalene (Wahlberg and Maibach, 1981, 1982; Fischer and Rystedt, 1985; Dooms-Goossens et al., 1986). In occupational exposure, only a minority of subjects develop pustular or acneiform dermatitis. Thus, the development of this type of irritant contact dermatitis appears to be dependent on both constitutional and chemical factors. Cosmetic dermatitis commonly assumes this morphology.

13.1.10 Exsiccation Eczematoid

Exsiccation eczematoid is seen mainly in elderly individuals during the winter months, when humidity is low. Patients suffer from intensive itching, and their skin appears dry with ichthyosiform scaling. The condition is thought to be due to a decrease in skin surface lipid and persistence of both peripheral and nonperipheral corneodesmosomes in the upper stratum corneum (Simon et al., 2001). In severe cases, a reduction of skin content of amino acid due to low profilaggrin biosynthesis was found (Horii et al., 1989).

13.1.11 Friction Dermatitis

This is sometimes seen on the hands and knees in the workplace, and results from frictional trauma. The syndrome has been characterized by Susten (1985).

13.2 EXTERNAL FACTORS

The variable clinical response to primary irritants is attributed to factors such as chemical characteristics of the irritants, physical nature, binding capacity, polarity, exposure time, cumulative effect with other irritants and environmental conditions.

13.2.1 Irritants

Many chemicals qualify as irritants when the exposing dose is high (Kligman and Wooding, 1967). Increasing the concentration of a compound may change the pattern of the response; sodium lauryl sulfate (SLS) and croton oil induce erythema with low concentrations and bullae with high concentrations (Bjornberg, 1968). Quantitative differences in the skin response to different concentrations of SLS were also noted (Agner et al., 1990; Aramaki et al., 2001; Loffler et al., 2001). It is believed that the biological effect of a substance depends on the amount of the molecules (molarity) rather than the total mass of the molecules (concentration) (Tupker, 2003). In addition, the absorbed dose may vary when the substance is suspended in different vehicles (Cooper, 1985; Gummer, 1985). The solubility of the irritant in the vehicle and the inherent irritancy of the vehicle have an impact on each reaction (Flannigan and Tucker, 1985).

13.2.2 Exposure

The effective tissue dose depends on application time and duration on and in the skin.

Long time of exposure and large volume increase penetration, thus, greater response may be expected (Aramaki, 2001). If exposure is repeated, the recovery from previous exposure(s) affects the subsequent response. Sometimes a shorter, repeated exposure leads to a lengthened recovery period (Malten and den Arend, 1978). This was demonstrated in experimental studies with dimethyl sulfoxide (DMSO), where intermittent application led to a different response as compared with one lengthened application (Lammintausta et al., 1988a). Skin may adapt to topical irritants through accommodation. A single exposure can induce resistance to subsequent exposures in the form of an adaptive downregulation of the inflammatory response or by changes in the stratum corneum lipids (Guin, 1991; Kawai, 1991; Wahlberg, 1992; Widmer et al., 1994; Heinemann, 2005). However, in a recent study adaptive hyposensitivity was not observed after long-term repetitive irritant exposure (Branco, 2005). These experimental observations are consistent with the multiple clinical appearances of cumulative irritant dermatitis.

13.2.3 Multiple Simultaneous Exposures

Simultaneous or subsequent exposure may lead to an additive effect and increased reaction, although each chemical alone would elicit only a minor reaction, or none (Fluhr et al., 2005a,c). However, subsequent exposure may lead to a decreased response. For instance, exposure to a detergent and then to a soap led to a weaker response than exposure to a detergent alone. The detergent was washed away by the subsequent soap exposure (Malten, 1981). Similarly, exposure to benzalkonium chloride, a cationic surfactant, and then to sodium dodecyl sulfate (SDS), an anionic surfactant, resulted in a milder irritant reaction (McFadden et al., 2000). However, when SLS and toluene were concurrently applied, significantly stronger effects were noted than twice daily application of SLS or toluene alone (Wigger-Alberti et al., 2000). No synergistic effect and limited additive effect were found with serial application of 0.5% SLS and *n*-propanol (Kappes et al., 2001). Tandem application of ascorbic acid, acetic acid, and NaOH

with SLS led to additive effects (Fluhr et al., 2004). In contrast, combined exposure to fruit acids and SLS did not enhance cumulative skin irritation *in vivo* (Schliemann-Willers et al., 2005). A "crossover" phenomenon between two distinct irritants has been suggested. The serial application of SLS retinoic acid (RA) caused considerably stronger erythema, more scaling, higher transepidermal water loss (TEWL) values, and decreased stratum corneum hydration than the serial application of RA/SLS or the individual components alone (Effendy et al., 1996; Ale et al., 1997). A pharmacological synergism or antagonism between the compounds may explain this phenomenon. Alternatively, the effects of one agent may result in a change in the kinetics of the percutaneous penetration of the other. Sometimes the outcome of multiple, subsequent, or simultaneous exposures is unexpected (Lammintausta et al., 1987a) and rules must be sought (Pittz et al., 1985).

13.2.4 Environmental Factors

Low humidity enhances irritability; testing the skin with irritants produced more and stronger reactions during the winter when the weather was cool, windy, and dry (Hannuksela et al., 1975; Agner and Serup, 1989; Basketter et al., 1996b; Leggat and Smith, 2006). Temperature may affect the skin response to irritants, warm temperatures are generally more damaging than cool temperatures. Warm temperature increased skin irritation induced by surfactant (Berardesca, 1995), and *in vitro* penetration of SLS was also increased with increasing the temperature (Emilson et al., 1993). Furthermore, it was shown that exposure to 43°C citral perfume led to primary irritation which continued even after 48 h, while the reaction to the same perfume at 23°C was reduced 3 h after exposure (Rothenborg et al., 1977). It is well known that water temperature influences the irritant capacity of a detergent. Higher ionic content and higher temperature were found to be determinative for the irritant potential (Clarys, 1997). Exposure to local heat sources together with detergents represents a common workplace situation. Warm airflow at different temperatures increased SLS-induced barrier disruption (Fluhr et al., 2005d). While, in a short-term model, cold temperatures were found to have a protective effect on the development of irritation (Fluhr et al., 2005b). Changes in temperature may be an important means for prevention of irritant contact dermatitis (Ohlenschlaeger, 1996). UVB diminished immediate reactions induced by phenol and DMSO and delayed reactions from SLS and DMSO (Larmi et al., 1989). Occlusion enhances stratum corneum hydration and often increases percutaneous absorption and acute irritation. In contrast, it compromises skin barrier function by impairing passive TEWL at the application site. Thus, skin reactions frequently become stronger when the chemical is applied under occlusion (van der Valk et al., 1989a), providing a humid environment that minimizes evaporation and makes the stratum corneum more permeable. Gloves and clothing increase the susceptibility for irritant dermatitis. Frequent changes of these articles is important to minimize the humid and occlusive environment. Occlusion alone may produce cytological damage to the skin that had been termed *hydration dermatitis* by Kligman. Stratum corneum lipids are implicated as an important determinant in water-retaining properties and the barrier function. A seasonal comparison of the total lipid amounts extracted from the stripped stratum corneum revealed an increased level in the summer, while the levels of ceramides were slightly increased in the winter compared with the summer (Yoshikawa et al., 1994).

TABLE 13.4
Common Airborne Irritants

Volatile substances
Acids and alkalis, ammonia
Cleaning products
Formaldehyde
Industrial solvents
Noncarbon required (NCR) paper
Epoxy resins
Foams (e.g., insulation foams in urea-formaldehyde process)
Powders
Aluminum
Anhydrous calcium silicate
Cement
Cleaning products
Metallic oxides
Particles
Tree-Sawing particles
Wool
Plastics, dry
Particles from plants
Stone particles in mining

13.2.5 Airborne Irritation

Airborne irritation dermatitis is located most commonly in exposed skin areas, such as the face, hands, and arms (Lachapelle, 1986). Volatile chemicals and dusts are common sources of exposure, but even sharp particles in dust may induce lesions (Table 13.4). Airborne irritation is a type of exposure in which physical sources of irritation frequently exacerbate the response with an additive influence. For instance, sunlight, wind, and cold air are additive to chemical exposure. Depending on the occupational situation, multiple environmental and occupational irritants may induce airborne irritation (Dooms-Goossens et al., 1986).

13.3 PREDISPOSING FACTORS

13.3.1 Methodological Aspects

Identification of subjects at risk for irritant dermatitis by screening tests is desirable to adjust the preventive measures. Individual susceptibility to chemicals has been studied by documenting the skin reactivity to model irritants, by measuring the intensity of the wheal created by DMSO, and the time required to raise a blister (minimal blistering time [MBT]) after cutaneous application of ammonium hydroxide solution

(Frosch, 1985). Stinging has been also studied with certain test substances (e.g., lactic acid, capsaicin) (Frosch and Kligman, 1982; Jourdain et al., 2005). Methods for quantifying inter and intraindividual differences in stratum corneum barrier function were also described. High correlation between subjects developing increased water loss and propensity for SLS damage after application of sodium hydroxide was found (Wilhelm et al., 1990), and recently a visual assessment method which determines subject irritant threshold, studying the relationship between SLS irritant threshold and TEWL measurements of normal skin and SLS patch tests, was described (Smith et al., 2004). An increased baseline TEWL in patients with acute and healed irritant contact dermatitis or eczema was also noted (Effendy et al., 1995; Kuzmina et al., 2003). Later on, an association between reactivity to an irritant and the likelihood of positive elicitation reactions to lower hapten concentrations was noted (Smith et al., 2002). These simple approaches provided a first step toward a preemployment test for irritant dermatitis potential. A genetic marker of irritant susceptibility in normal individuals was also described. In this study, an association of tumor necrosis factor (TNF) α gene polymorphism at position –308 with susceptibility to irritant dermatitis was noted (Allen et al., 2000). However, despite important steps taken in the investigation of the pathogenesis of irritant contact dermatitis, no experimental design has proved entirely successful for the clinical evaluation of individual susceptibility.

The main factors that influence individual proclivity are age, race, sex, site, history of dermatitis, and genetic background.

13.3.2 Regional Anatomic Differences

Skin permeability is variable in different skin sites, being generally greatest in thin skin areas (Cronin and Stoughton, 1962; Feldmann and Maibach, 1967a,b; Wester and Maibach, 1985, 1989; Tur et al., 1985). Corresponding association between permeability, skin thickness, and skin irritation is expected, but direct correlation is lacking. Regional variations were noted in the whealing response to DMSO, and in the time for blister formation (MBT) after topical ammonium hydroxide application (Frosch and Kligman, 1982). Both tests showed the mandibular area to be the most reactive, followed by the upper back, forearm, lower leg, and palm. With DMSO whealing, the forehead was more sensitive than the back, the antecubital area reaction preceded that of the rest of the upper extremity, and the wrist was more sensitive than the leg. Patch tests with the irritant benzalkonium chloride and a number of allergens produced maximal reactivity in the upper back (Magnusson and Hersle, 1965), particularly in the middle scapula (Flannigan et al., 1984). The greater reactivity may be related to pressure in this area when sleeping (von Hornstein and Kienlein-Kletschka, 1982; Gollhausen and Kligman, 1985). TEWL measurements after exposure to SLS revealed the thigh to be the most vulnerable site followed by the upper arm, abdomen, upper back, dorsal and volar forearm, postauricular, and ankle, with the palm as the least vulnerable location (Cua et al., 1990). Recently, human scalp irritation was compared to that of the arm and back. Results indicated that the back was most sensitive to SLS treatment (Zhai et al., 2004). Later on regional and age-related differences in the human face were examined in two age groups. In both age groups, one with an average age of 25.2 ± 4.7 years and another with an average age of 73.7 ± 3.9 years some significant differences between the regions of the face were detected. The younger group showed higher changes in TEWL than the older group in all the areas studied, but only in the chin and nasolabial area were the differences statistically significant (Marrakchi and Maibach, 2006). When the volar surface of the forearm was studied the potential for irritation increased from the wrist to the cubital fossa (van der Valk, 1989b). Vulvar skin was significantly more reactive than the forearm to benzalkonium chloride (17%) and maleic acid (20%) (Britz and Maibach, 1979; Oriba et al., 1989). No differences were found between vulvar and forearm skin when SLS was applied at various concentrations (Elsner et al., 1990). However in clinical occupational dermatology, it is often noted that male genitalia are affected in occupational irritant dermatitis. Since cutaneous irritant responses to various irritants might be mediated by distinctly different pathophysiological pathways, regional susceptibility to diverse irritants vary accordingly (Patrick et al., 1985). Additionally, stratum corneum barrier properties have been associated with stratum corneum lipid composition, regions with higher neutral lipids and lower sphingolipids are generally associated with superior barrier properties (Lampe et al., 1983). Certain "inherent" differences between different skin sites in irritation reactivity may also exist.

13.3.3 Age

The threshold for skin irritation is decreased in babies, who develop dermatitis from irritation that usually does not occur in adult skin (Jordan and Blaney, 1982). Except for structural and functional immaturity of infant's skin, other factors (intestinal *Candida albicans,* low frequency of diaper changes) are contributory (Seymour et al., 1987). Children below the age of 8 years are generally considered more susceptible to skin irritation (Mobly and Mansmann, 1974; Epstein, 1971; Fisher, 1975), irritation susceptibility gradually decreases after this age. Maibach and Boisits (1982) define this database; unfortunately, despite extensive chemical exposure of infants and children, experimental evidence is lacking because of methodological problems and limited data. Studies in newborn infants showed high and variable TEWL in the first 4 h after birth settling to a constant level thereafter (Rutter, 1978), and newborn term infants have less TEWL than adults (Cunico et al., 1977). No differences in baseline TEWL were demonstrable between young and old individuals (Thune et al., 1988; Roskos, 1989; Wilhelm and Maibach, 1989). However, elderly subjects reacted to skin irritants less sharply and more slowly than younger individuals (Grove et al., 1981; Lejman et al., 1984; Schwindt et al., 1998; Robinson, 2002; Marrakchi and Maibach, 2006), the difference was particularly significant in sites characterized by low TEWL under basal conditions

TABLE 13.5
Cutaneous Sodium Lauryl Sulfate Irritation Potential: Age and Regional Variability

Region	Young	Old	Significance
Forehead			
Control	7.1 ± 1.1	4.8 ± 1.7	NS
SLS	15.8 ± 2.1	14.1 ± 5.0[a]	NS
Upper arm			
Control	3.8 ± 0.7	1.8 ± 0.6	$p < .05$
SLS	17.0 ± 5.2[a]	4.6 ± 1.6[a]	$p < .05$
Volar forearm			
Control	5.0 ± 1.4	2.3 ± 0.8	NS
SLS	13.3 ± 4.5	5.3 ± 1.9[a]	NS
Dorsal forearm			
Control	3.8 ± 0.7	2.1 ± 0.8	NS
SLS	11.6 ± 2.8[a]	4.2 ± 1.5[a]	$p < .05$
Postauricular			
Control	6.1 ± 0.8	6.6 ± 2.3	NS
SLS	11.4 ± 2.3[a]	11.6 ± 4.1[a]	NS
Palm			
Control	31.0 ± 7.7	26.2 ± 9.3	NS
SLS	26.9 ± 4.0	22.5 ± 8.0	NS
Abdomen			
Control	5.4 ± 0.9	1.9 ± 0.7	$p < .01$
SLS	20.0 ± 4.7[a]	5.1 ± 1.8[a]	$p < .01$
Upper back			
Control	5.6 ± 1.2	3.3 ± 1.2	NS
SLS	18.2 ± 3.5[a]	6.6 ± 2.3[a]	$p < .02$
Thigh			
Control	5.1 ± 1.6	2.2 ± 0.8	NS
SLS	24.6 ± 7.6[a]	7.1 ± 2.5[a]	$p < .05$
Ankle			
Control	6.2 ± 1.7	2.5 ± 0.9	NS
SLS	6.5 ± 1.8	3.7 ± 1.3	NS

Note: SLS–irritant patch-test reactions: Transepidermal water loss measurements (TEWL, g/m^2/h) SLS, sodium lauryl sulfate.

[a] Differences were compared between control and SLS-treated sites. NS, not significant ($p < .05$).

Source: Cua, A.B., Wilhelm, K.P. and Maibach, H.I., Br. J. Dermatol., 123, 607–613, 1990.

(Cua et al., 1990) (Table 13.5). When pre and postmenopausal women were compared, age-related differences were apparent in the forearm skin, but not the vulva (Elsner et al., 1990). A corresponding alteration occurred with regard to cutaneous reactivity to allergens. With ammonium hydroxide skin tests, older subjects had a shorter reaction time (MBT), whereas the time needed to develop a tense blister was longer (Frosch and Kligman, 1977), and a longer time was also needed for the absorption of a wheal elicited by saline injection (Kligman, 1976). Age-associated alterations in skin reactivity may be related to altered cutaneous penetration, although contradictory results have been reported (Christophers and Kligman, 1965; Tagami, 1971; DeSalva and Thompson, 1965; Guy et al., 1985; Roskos et al., 1990). Alterations in structural lipids (Elias, 1981), in cell composition (Gilchrest et al., 1982) and renewal (Baker and Blair, 1968; Roberts and Marks, 1980), are reported in association with structural alteration (Montaga and Carlisle, 1979; Holzle et al., 1986). Finally, the effects of stratum corneum hydration on irritancy potential is probably noncontributory, baseline capacitance did not differ between age groups on most regions (Cua et al., 1990).

13.3.4 Race

Ethnic differences in skin physiology and pathophysiology exist (Berardesca and Maibach, 1988c; Berardesca et al., 1991b; Berardesca and Maibach, 1996). Results based on traditional visual grading suggest that blacks have less-irritable skin than Caucasians (Weigand and Gaylor, 1974; Andersen and Maibach, 1979). Use of alternative evaluative techniques, including laser Doppler flowmetry (LDF), and TEWL tend to support this conclusion. However, when SLS was applied to untreated, preoccluded, and predelipidized skin, blacks had higher TEWL levels than Caucasians in the preoccluded state (Berardesca et al., 1988a). This finding contradicts the hypothesis that blacks are less susceptible to irritation. However, no significant differences were noted between black and Caucasian subjects in LDV measurements after application of methyl-nicotinate (Gean et al., 1984: Guy et al., 1985), or irritation with SLS (Berardesca et al., 1988a). Similarly, fluorescence excitation spectroscopy demonstrated no differences in the hyperproliferative response after irritant exposure, and indicated similar kinetics for 15 Caucasian and 15 African-American patients tested (Astner et al., 2006). Baseline TEWL measurements were significantly higher in Asian and black subjects compared with white subjects. No baseline differences were seen between black and Asian subjects (Kompaore et al., 1993). No difference was found in this parameter when African black subjects were compared with white European subjects or among other racial groups studied (DeLuca et al., 1983; Pinnagoda et al., 1990). Blacks and Hispanics showed higher TEWL responses to SLS (Berardesca et al., 1988a,b) and *in vitro* TEWL differences between blacks and whites have also been reported (Wilson et al., 1988). In a similar study, Chinese have been found to be more sensitive than Malaysians but no significant differences were found between Chinese and Indians or between Malaysians and Indians (Goh and Chia, 1988). Rapaport using a 21-day cumulative irritation test protocol found among the Japanese subjects generally greater cumulative irritation scores for 13 of the 15 irritants tested (Rapaport, 1984). Subsequent studies using acute and cumulative skin irritation tests in Caucasian and Asian populations failed to confirm a consistent trend. No significant differences were seen between Caucasian and Chinese subjects exposed to chemicals of varying irritation potency under a 4-h patch test (Robinson, 2000). Japanese subjects showed a tendency to respond faster than the Caucasian subjects at several chemical exposure time points in the acute irritation test, and in the cumulative irritation test only with the lowest concentration of SDS (Robinson, 2000). When the same study was repeated among

the same populations, no differences were seen in the acute and cumulative skin irritation responses between the Japanese and Caucasian subjects. However, Chinese subjects showed a heightened response to acetic acid after 4 h of exposure, and a slower and less-severe response in the cumulative irritation test compared to Caucasian and Japanese subjects (Robinson, 2000). Later on, Caucasian and Japanese women were tested with a range of irritant materials. The acute irritation response tended to be greater in the Japanese panel, and this was statistical significant with the stronger irritants. In the cumulative irritation test the Japanese compared to Caucasian panellists showed a higher response only with the weaker irritants, however, the results were not significant (Foy et al., 2001). When Japanese subjects were tested to cosmetic products and topical tretinoin cream, a high level of intolerance was also noted (Ishihara et al., 1986; Tadaki et al., 1993). An international dose–response study with the anionic surfactant SLS at different concentrations was conducted under Unilever's direction. In this study, the German population tended to be the most responsive and the Asian population was not more reactive than the Caucasians (Basketter et al., 1996b). Similarly, Robinson analyzed skin reactivity in different human subpopulations. Results were compiled from nine acute irritation patch-test studies, conducted at three test facilities over a 5-year period. For three irritant test chemicals, 20% SDS, 100% decanol, and 10% acetic acid, an increased reactivity for Asian versus Caucasian subjects was noted (Robinson, 2002). However, studying populations in different geographic locations and at different times of the year creates difficulties in the interpretation of the data. The different studies indicate that there is little evidence of statistically significant differences in the irritant response among Caucasian, black, and Asian groups, and there is no consensus on whether race contribute as an endogenous factor in the development of irritant contact dermatitis.

13.3.5 Gender

There is a common perception that women are more prone to skin irritation than men (Agrup, 1969; Lantinga et al., 1984; Rystedt, 1985), yet, when four irritants were compared, male subjects were found to be directionally or significantly more reactive than females to each of the irritants tested (Robinson, 2002). In line with this experimental observation is the result of a multicenter study of the German contact dermatitis research group, where irritant reaction to SLS was somewhat increased in males (Uter et al., 2004). However, differences between males and females were not experimentally documented in most studies (Bjornberg, 1975; Lammintausta et al., 1987b; Goh and Chia, 1988; Meding, 2000). The increased occurrence of irritant contact dermatitis in females may be related to the more extensive exposure to irritants and wet work. A minimal relationship between gender and constitutional skin irritability is supported by the fact that the female preponderance in the irritant contact dermatitis populations does not hold true for all geographic areas (Olumide, 1987).

13.3.6 Previous and Preexisting Skin Diseases

It has been suggested that existing dermatitis irrespective of which type may enhance skin reactivity to various irritants (Bjornberg, 1968). Skin response to SLS was statistically significantly increased in atopics compared to controls, when evaluated by visual scoring and skin thickness (Agner, 1991). Increased water loss with detergent patch tests and several other irritants was also reported (van der Valk et al., 1985; Tupker et al., 1990; Goh, 1997). However, when 20% SDS, 35% cocotrimethyl ammonium chloride, and 10% hydrochloric acid were tested, a higher reaction of atopic skin was found only to 20% SDS, but not to 35% cocotrimethyl ammonium chloride and 10% hydrochloric acid (Basketter et al., 1996a). Different concentrations of SLS were also tested, a higher percent of positive results and a significantly greater intensity of response were noted in the atopic dermatitis group than in controls; the same result was demonstrated in atopic allergic rhinitis patients without dermatitis (Nassif et al., 1994). The responsiveness to SLS was not increased in patients with chronic or healed dermatitis (Agner, 1991). Later on, the relative reactivity of an apparently normal skin in atopic and nonatopic groups was studied. SLS was applied at a range of concentrations and exposure times. At various time points, the irritation response was measured by visual assessment, chromametry, LDF, and TEWL. Using all of the methods of assessment, the reactions in atopics were similar to or a little less than those seen in nonatopics (Basketter et al., 1998). Hannuksela and Hannuksela data suggested that different methods of application, like open application and plastic chamber, may produce dichotomous results (Hannuksela and Hannuksela, 1995). The enhanced irritant susceptibility in atopic dermatitis at least in part is explained by constitutional factors. Patients with atopic dermatitis are alleged to have defective skin barrier function, both in irritated and normal looking skin. Itchy and dry atopic skin has been connected with an increased risk for developing hand dermatitis (Lammintausta and Kalimo, 1981; Rystedt, 1985). Reduced capacity to bind water has been related to atopic skin (Werner et al., 1982), which in noneczematous sites demonstrates greater TEWL than does nonatopic skin, and stratum corneum water content may even be increased (Finlay et al., 1980; Al Jaber and Marks, 1984; Gloor et al., 1981). Furthermore, keratinocytes of atopic dermatitis patients after a nonspecific stimulation produced higher levels of TNF-α and interleukin (IL)-1 compared to nonatopics (Pastore et al., 1998). Ichthyosis vulgaris is sometimes seen in association with atopic dermatitis; in ichthyosis vulgaris, patients' irritant reactivity has been shown to be increased to alkali irritants (Ziierz et al., 1960). Seborrheic skin has not been shown to possess increased susceptibility to skin irritants; reports and interpretations are contradictory (Holland, 1958; Vickers, 1962; von Hornstein et al., 1986). However, exposure to SLS induced significantly greater blood flux in patients with seborrheic dermatitis (Cowley and Farr, 1992). Clinical experience suggests that some increased irritability is associated with a seborrheic constitution in

certain subjects. This may be true in certain geographic areas, where environmental humidity is low in the winter in relation to the cold temperatures.

Different methods have been used in studies on skin irritability in psoriatic individuals. These studies revealed decreased and increased irritant reactivity (Kingston and Marks, 1983; Maurice and Greaves, 1983; Lawrence et al., 1984; MacDonalds and Marks, 1986) when anthralin (dithranol) irritancy was the main interest. Psoriatic skin is particularly irritable in certain individuals (Epstein and Maibach, 1985), and the development of psoriatic lesions in irritation sites (Koebner phenomenon) is often seen. In the presence of eczema, the threshold for skin irritation is decreased (Mitchell, 1981; Bruynzeel et al., 1983; Bruynzeel and Maibach, 1986; Agner, 1991). A whole-body examination of employees sometimes reveals nummular lesions or other constitutional eczema symptoms. Such a clinical finding may suggest increased skin irritability in different locations. Pompholyx (dyshidrosis) type dermatitis is harmful. As a constitutional eczema, it probably increases skin irritability in general. These patients often have difficulty wearing gloves, since phompholyx is made worse by occlusion. A history of contact dermatitis may be important when susceptibility to irritant contact dermatitis is evaluated (Nilsson et al., 1985; Lammintausta et al., 1988a). Although increased irritability exerted by the preexisting dermatosis was hard to demonstrate, further improvement of methodological equipment in the bioengineering industry should make this possible (Lammintausta et al., 1988a; Santucci et al., 2003).

13.3.7 Genetic Background

Irritant dermatitis is a multifaceted disease and the mechanisms underlying susceptibility to skin irritants are not clearly understood. However, a growing body of evidence suggests that immunologic mechanisms may also in part underlie the pathogenesis of irritant contact dermatitis. As different irritants with different chemical properties of molecules produce different effect on epidermal structures, it seems likely that there are many routes by which irritant dermatitis may arise. However, all irritants bear in common the same pathophysiological changes including skin barrier disruption by chemical stimuli or mechanical trauma, cellular epidermal damage and release of proinflammatory mediators particularly cytokines, all of which are interlinked. Disruption of the barrier leads to release of cytokines such as IL-1 α, IL-1 β, and TNF-α. Furthermore, when the barrier is disrupted, the entry of chemicals into the epidermis is facilitated, leading to structural changes in keratinocytes and further cytokine release. Among the proinflammatory cytokines which have been found to be released are IL-1 α, TNF-α, IL-6, IL-8, granulocyte–macrophage colony-stimulating factor (GM-CSF), and T-cell-derived cytokines, including IL-2 and interferon-γ (Wood et al., 1992; Ulfgren et al., 2000). As TNF-α plays a key role in inflammation, it seems likely that factors affecting the production of TNF-α may account for the degree of the irritation response and the interindividual variations. Allen et al. investigated the relationship between polymorphism in the TNF-α-chain gene and responses to irritants. In this study, an association of TNF-α gene polymorphism at position −308 with susceptibility to irritant dermatitis was noted (Allen et al., 2000). This is the first description of a genetic marker for irritant susceptibility in normal individuals, and further studies to support this result are needed.

13.4 SUMMARY

The mechanisms by which various chemicals elicit irritant dermatitis are not clearly understood. While strong irritants quickly elicit signs and symptoms of dermatitis, weak irritants may not. The severity of the dermatitis is highly variable and depends on the amount and strength of the irritant, length and frequency of exposure, environmental factors, and skin susceptibility. The main factors that influence individual proclivity are age, race, sex, site, history of dermatitis, and genetic background. Thus, age-associated alterations in cutaneous reactivity are expected; however, the subject requires more investigation. Conflicting findings have been reported comparing skin response to irritants in Caucasian, black, and Asian groups, and there is no consensus on whether race contribute as a factor in the development of irritant contact dermatitis. There is a common perception that women are more prone to skin irritation than men, yet, in recent studies male sex was identified as a relatively weak but significant risk factor for the occurrence of irritant dermatitis. It has been suggested that preexisting dermatitis irrespective of which type may enhance skin reactivity to various irritants. However, the role of preexisting dermatitis in the response to irritants resulted quite marginal in most of the studies. It seems that regional variations in skin irritation do exist and further studies to support these result are needed. Identification of subjects at risk for irritant dermatitis by screening tests is desirable. A recent description of a genetic marker of irritant susceptibility in normal individuals seems promising.

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14 Systemic Contact Dermatitis

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14.1 INTRODUCTION

Systemic contact dermatitis is an inflammatory skin disease that may occur in contact-sensitized individuals when these persons are exposed to the hapten orally, transcutaneously, intravenously, or by inhalation. The entity can be present with clinically characteristic features or be clinically indistinguishable from other types of contact dermatitis. Contact sensitization to ubiquitous haptens is common. In a recent Danish population-based study, 15.2% reacted to one or more of the haptens in the standard patch-test series (Nielsen et al., 2001). The total number of individuals at risk of developing a systemic contact dermatitis reaction is therefore large. Systemic contact dermatitis from medicaments is a well-established entity. There is increasing evidence for similar reactions from plant derivatives and metals such as nickel (Hindsén et al., 2001).

The first description of systemic contact dermatitis can probably be ascribed to the pioneering British dermatologist Thomas Bateman (Shelley and Crissey, 1970). His description of the mercury eczema called eczema rubrum is similar to what we today describe as the baboon syndrome.

Eczema rubrum is preceded by a sense of stiffness, burning, heat, and itching in the part where it commences, most frequently the upper and inner surface of the thighs and about the scrotum in men, but sometimes it appears first in the groins, axillae or in the bends of the arms, on the wrists and hands, or on the neck.

In this century, the systemic spread of nickel dermatitis was described by Schittenhelm and Stockinger (1925) in Kiel. By patch testing nickel-sensitive workers with nickel sulfate, they observed the spread of dermatitis and flares in the original areas of contact dermatitis. Similar clinical features have been seen in large groups of carefully evaluated nickel-sensitive patients Marcussen (1957); Calnan (1956). The literature on systemic contact dermatitis is now comprehensive. Reviews include Veien et al. (1990), Menné et al. (1994), and Veien and Menné (2006); Fisher (1986); Cronin (1980).

14.2 IMMUNOLOGY/MECHANISM

Systemic contact dermatitis may start a few hours or 1–2 days after experimental provocation, suggesting that more than one type of immunological reactions are involved. The local flare-up reaction has been studied experimentally in both humans and in animals. Christensen et al. (1981) studied flare-up reactions in 4- to 7-week-old positive nickel patch tests in five nickel-sensitive patients after oral provocation with 5.6 mg nickel. The histology was that of acute dermatitis. Direct immunofluorescence examination for deposits of IgG, IgA, IgM, complement 3, and fibrinogen was negative. A few sensitized T cells can remain in the skin for months (Scheper et al., 1983; Yamashita et al., 1989). Systemic exposure to haptens can activate sensitized T cells in sites of previous contact dermatitis and initiate the inflammatory response.

A patient had parthenium dermatitis that was aggravated during stays in an area of India where parthenium grew profusely. Inhalation of material from fresh parthenium plants resulted in aggravation of the dermatitis without respiratory symptoms (Mahajan et al., 2004).

The investigation of lymphocyte subsets in the gastrointestinal mucosa and in blood before and after oral challenge with nickel in nickel-sensitized women showed a reduction of CD4+ cells, CD4+CD45Ro+ cells, and CD8+ cells in the peripheral blood of women with evidence of systemic contact dermatitis. Oral challenge with nickel induced maturation of naive T cells into memory cells that tended to accumulate in the intestinal mucosa (di Gioacchino, 2000).

Jensen et al. (2004) found a reduction of the number of CLA+ CD45Ro+ CD3 and CLA+ CD45Ro+ CD8 but not CLA+ CD45Ro+ CD4 in the peripheral blood of nickel-sensitive patients after oral challenge with nickel.

Following the removal of a metal joint prosthesis containing cobalt, CD4 T-cell clones reacted to cobalt but not to nickel (Thomssen et al., 2001).

Möller et al. (1998) challenged 10 gold allergic patients with an intramuscular dose of gold and saw a flare-up of 1-week-old gold patch-test reactions in all of them. Five also experienced a maculopapular rash, and four had transient fever. Plasma levels of TNF-α, IL-1 ra, and sTNF-R1 and C-reactive protein were increased, particularly in those with fever.

In a later study of 20 gold and 28 nickel-allergic patients challenged orally with nickel and gold in a double-blind, double-dummy fashion, 3 of 9 nickel-sensitive patients reacted to 2.5 mg nickel, while none reacted to gold. Six of 10 gold-allergic patients reacted to 10 mg gold sodium thiomalate, none of them reacted to nickel. TNF-R1 was increased in the plasma of nickel-sensitive patients challenged with nickel, while TNF-R1, TNF-α, and IL-1 were increased in gold-sensitive patients challenged with gold (Möller et al., 1999a).

In a study of 42 patients with systemic contact dermatitis from *Toxicodendron*, it was suggested that a toxic rather than a specific immune reaction might be responsible (Oh et al., 2003).

The mechanism behind skin symptoms unrelated to previous contact dermatitis sites has been minimally evaluated. Veien et al. (1979) investigated 14 patients with positive nickel patch tests and severe dermatitis. All were challenged orally with 2.5 mg nickel. After 6–12 h, five developed widespread erythema. No clinical dermatitis developed in the erythematous areas. In a passive immunodiffusion assay, three of the five demonstrated precipitating antibodies in their sera against a nickel–albumin complex. The same phenomenon was observed by Polak and Turk (1968a,b) in chromium-sensitized guinea pigs. As in humans, the response started 6–8 h after a chromate injection. Histopathology 24 h after the challenge showed a marked dilatation of the capillaries in the upper dermis. The authors suggested that circulating immune complexes were the triggering mechanism.

Van Hoogstraten et al. (1992) demonstrated antigen-specific tolerance to nickel and chromate in guinea pigs. Administration of the allergens to the oral mucosa proved the most effective means of inducing tolerance. Prior to this study, the same author conducted a retrospective clinical study of the risk of nickel sensitization from pierced ears in patients who did or did not wear dental braces. It was shown that fewer cases of nickel sensitization were seen when dental braces were fitted before ear piercing than when the braces were fitted after ear piercing (Van Hoogstraten, 1991). These findings have been confirmed by Mørtz in a study of 13–15-year-old girls (Mørtz, 1999).

It has also been shown that giving repeated oral or sublingual doses of nickel to nickel-sensitive patients gradually reduces the severity of flares of dermatitis seen after oral challenge with nickel (Sjövall et al., 1987); Morris (1998); Panzani et al. (1995) or reduces circulating lymphocytes reacting to nickel (Bagot et al., 1999). It is possible that the repeated doses of nickel decrease the intestinal absorption of the metal (Santucci et al., 1994).

Hyposensitization to poison ivy was observed in 9 of 13 poison ivy-sensitive workers who were exposed to dust from cashew nut shells. The route of hyposensitization was thought to be gastrointestinal from swallowed dust (Reginella et al., 1989).

A patient was successfully hyposensitized to *Parthenium hysterophorus* by the oral route of exposure (Srinivas et al., 1988).

Twenty parthenium-sensitive patients were hyposensitized orally for 12 weeks with ether extracts of parthenium. Fourteen completed the procedure and improved. Six stopped due to aggravation of their dermatitis. Seven of the fourteen were followed for a year. Four of them had recurrences within 2 months (Handa et al., 2001).

Two patients with chrysanthemum dermatitis were successfully hyposensitized using chrysanthemum juice for 21 days. Aggravation of the dermatitis was seen initially in both patients. The patients remained clear of dermatitis for more than 2 years (Mori et al., 2000).

14.3 CLINICAL FEATURES

The clinical symptoms related to systemic contact dermatitis are summarized in Table 14.1. The symptoms usually appear exclusively on the skin, but general symptoms are occasionally

TABLE 14.1
Clinical Aspects of Systemic Contact Dermatitis Reactions

Dermatitis in areas of previous exposure
Flare-Up of previous dermatitis
Flare-Up of previously positive patch-test sites
Skin symptoms in previously unaffected skin
Vesicular hand eczema
Flexural dermatitis
The baboon syndrome
Maculopapular rash
Vasculitis-like lesions
General symptoms
Headache
Malaise
Arthralgia
Diarrhea and vomiting

seen. Knowledge of the clinical symptoms stems from clinical observations and experimental oral challenge studies.

Flare-up reactions in the primary site of dermatitis or previously positive patch-test sites raise the suspicion of systemic contact dermatitis (Ekelund and Möller, 1969; Christensen and Möller, 1975; Menné and Weismann, 1984; Hindsén et al., 2001). Flare-up of previously positive patch-test sites following ingestion of the hapten is a fascinating and specific sign of systemic contact dermatitis. It is seen in relation to systemic contact dermatitis from medicaments and in experimental oral provocation studies. Such flares of patch-test sites have not been a feature of the clinical spectrum of systemic contact dermatitis. This symptom is hapten specific and can be seen years after the original patch testing. Christensen et al. (1981) and later Hindsén et al. (2001) examined the specificity of this symptom in nickel-sensitive individuals. Positive patch tests to nickel and to the primary irritant sodium lauryl sulfate were made on previously unaffected skin areas. After several weeks, the individuals were given an oral nickel dose. A flare of dermatitis was seen at the nickel patch-test site, but not at the site of irritant dermatitis.

Vesicular hand eczema (pompholyx or dyshidrotic eczema) (Veien and Menné, 2000), a pruritic eruption on the palms, volar aspects and sides of the fingers, and occasionally the plantar aspects of the feet, presents with deep-seated vesicles and sparse or no erythema. If the distal dorsal aspects of the fingers are involved, transversal ridging of the fingernails can be a consequence. Recurrent, vesicular hand eczema is a common clinical manifestation of hand eczema and can have many different causes. It is a frequent symptom seen in systemic contact dermatitis.

Erythema or a flare of dermatitis in the elbow or the knee flexures is a common symptom of systemic contact dermatitis (Wintzen et al., 2003). It is difficult to distinguish from the early lesions of atopic dermatitis. Flexual psoriasis can be a Köbner phenomenon associated with systemic contact dermatitis.

The baboon syndrome (Andersen et al., 1984) is a well-demarcated eruption on the buttocks, in the genital area and in a V shape on the inner thighs with a color ranging from dark violet to pink. It may occupy the whole area or only part of it. Nakayama et al. (1983) described the same syndrome as mercury exanthema. Lerch and Bircher (2004) added acute, generalized exanthematous pustulosis to the syndrome. Based on case stories, the patients may have had systemic contact dermatitis. Even extensive patch testing fails to confirm the diagnosis of systemic contact dermatitis in some patients who present with features of the baboon syndrome.

A nonspecific maculopapular rash is often part of a systemic contact dermatitis reaction. Even cases of vasculitis presenting as palpable purpura have been seen (Veien and Krogdahl, 1989).

In relation to oral provocation with nickel or medicaments, general symptoms such as headache and malaise have occasionaly been seen in sensitized individuals. In neomycin- (Ekelund and Möller, 1969) and chromate-sensitive patients (Kaaber and Veien, 1977), oral provocation with the hapten has caused nausea, vomiting, and diarrhea. A few patients have complained of arthralgia. The available information on the general symptoms observed in relation to the systemic contact dermatitis reaction is anecdotal and deserves systematic documentation.

14.4 MEDICAMENTS

14.4.1 Antibiotics

Neomycin and bacitracin are widely used topical antibiotics. Contact allergy to these compounds is particularly frequent (4–8%) in patients with leg ulcers. Ekelund and Möller (1969) challenged 12 leg-ulcer patients sensitive to neomycin with an oral dose of the hapten. Ten of the twelve had a reaction. Five had flares of their original dermatitis; six had flares at the sites of previously positive patch tests. Three developed vesicular hand eczema for the first time. Four experienced various gastrointestinal symptoms. Some surgeons use oral neomycin prior to colon surgery. Even if neomycin is poorly absorbed from the gastrointestinal tract, severe systemic contact dermatitis might occur in neomycin-sensitive individuals (Menné and Weismann, 1984).

Contact sensitivity to penicillin was previously common, and flares of dermatitis have been seen in sensitized persons following exposure to traces of penicillin in milk (Vickers et al., 1958). Contact sensitivity and systemic contact dermatitis caused by penicillin can still occur after the topical use of the drug in the middle ear, in the peritoneum during abdominal surgery (Andersen et al., 1984), or after occupational exposure. Tagami et al. described a patient with toxic epidermal necrolysis after the systemic administration of ampicillin and reviewed 10 other patch-test proven cases of dermatitis of similar morphology caused by various medications (Tagami et al., 1983).

Penicillin, ampillin/amoxicillin, and erythromycin have been described as causes of systemic contact dermatitis with baboon-like clinical features (Llamazares, 2000; Goossens et al., 1997).

The baboon syndrome caused by drugs was reveiwed by Hausermann et al. (2004). Approximately 100 cases were reviewed. Most cases were caused by antibiotics.

Systemic contact dermatitis was seen in nurses occupationally sensitized to streptomycin (Wilson, 1958) when attempts were made to induce tolerance by subsequent systemic exposure to the drug.

During World War II, sulfonamides were used extensively for the treatment of wounds. Later systemic use of sulfonamides in sensitized individuals caused dose-dependent flares of dermatitis. The persons who had the most pronounced reactions were those most sensitive to sulfonamides (Park, 1943).

Systemic contact dermatitis caused by pristinamycine and gentamicin has also been described (Bernard et al., 1988; Ghadially and Ramsay, 1988).

14.4.2 Antihistamines

The pharmacological effectiveness of topically applied antihistamines is questionable. Antihistamines derived from ethanolamine and ethylenediamine are the most common contact-sensitizing antihistamines in the United States

(Fisher, 1976). Ethylenediamine-based antihistamines may elicit systemic contact dermatitis in patients sensitized to ethylenediamine. Aminophylline, which contains theophylline and ethylenediamine, may elicit reactions in ethylenediamine-sensitized patients (Provost and Jilson, 1967; Guin et al., 1999; Walker and Ferguson, 2004).

Much of the knowledge in this field is based on anecdotal therapeutic accidents. In view of the large number of persons who are contact sensitized to ethylenediamine, incidents of systemic contact dermatitis to ethylenediamine derivatives must be considered rare.

14.4.3 *para*-Amino Compounds

Sidi and Dobkevitch-Morrill (1951) studied cross-reactions between *para*-amino compounds. Systemic reactions were seen after oral challenge with procaine in sulfonamide-sensitive patients, after challenge with *p*-aminophenylsulfamide in procaine-sensitive patients, and after challenge with *p*-aminophenylsulfamide and procaine in *p*-phenylenediamine-sensitive patients.

Oral challenge with the sulfonyl urea hypoglycemic drugs in patients sensitized to *para*-amino compounds (sulfanilamide, *para*-phenylenediamine, and benzocaine) resulted in flare-up reactions in sulfanilamide-sensitive patients, but not in *para*-phenylenediamine and benzocaine-sensitive patients (Table 14.2). Oral challenge with tartrazine (20 mg) and saccarine (150 mg) in patients sensitized to *para*-amino compounds and sulfonamide did not produce any flare-up reactions (Angelini and Meneghini, 1981; Angelini et al., 1982).

14.4.4 Corticosteroids

Contact allergy to glucocorticoids is not uncommon in patients with eczematous skin diseases (Lauerma, 1992). The frequency seems to vary from center to center depending on local prescribing habits, degree of patient selections, and the diagnostic method used.

Patch testing with topical corticosteroids has not yet been standardized with regard to patch-test concentrations and vehicles. Intradermal testing may offer additional diagnostic possibilities.

Patients sensitized to hydrocortisone may react with systemic contact dermatitis when provoked orally with 100–200 mg hydrocortisone (Lauerma et al., 1991; Torres et al., 1993). These authors also investigated whether cortisol produced in the adrenals (i.e., hydrocortisone) could provoke systemic contact dermatitis. In a placebo-controlled study, a patient was challenged with an adrenocorticotropic hormone (ACTH) stimulation test. A skin rash similar to that seen after oral hydrocortisone developed after 8 h.

Räsänen and Hasan (1993) studied five patients who developed rashes when treated with systemic or intralesional hydrocortisone. They recommend patch testing and intradermal testing to make the diagnosis of systemic hydrocortisone sensitivity and, if these tests fail, an oral challenge.

Whitmore (1995) reviewed 16 studies with a total of 24 patients who had systemic contact dermatitis from corticosteroids. Typical clinical features were exanthema, localized dermatitis, generalized dermatitis, and purpura. Onset was often hours to days following ingestion of the corticosteroids.

As a part of her thesis on corticosteroid allergy, Isaksson (2000) challenged 15 budesonide-sensitive patients with 100 and 800 μg budesonide or placebo by inhalation. Four of seven challenged with budesonide had reactivation of previously positive patch-test sites as well as papular exanthema or a flare-up of previous dermatitis.

Pirker et al. (2003) saw an anaphylactoid reaction after inhalation of budesonide in a patient who was contact sensitized to budesonide. In another study, a betamethasone-sensitive patient developed baboon syndrome after the oral administration of betamethasone (Armingaud et al., 2005).

TABLE 14.2
Oral Challenge with Sulfonyl Urea Hypoglycemic Drugs in Sulfanilamide-Sensitive Patients

Substance	Allergen Dose (mg)	Duration of Treatment	Response
Carbutamide	500	Single exposure	7/25
Tolbutamide	500	Single exposure	3/11
Chlorpropamide	500	Single exposure	1/20

14.4.5 Miscellaneous Medications

Antabuse (tetraethylthiuram disulfide) is of particular interest, since it can cause systemic contact dermatitis in three ways. Antabuse is used in the manufacture of rubber as a fungicide and in the treatment of chronic alcoholism. In patients sensitized to thiurams from the use of rubber gloves, systemic exposure to Antabuse can give rise to systemic contact dermatitis (Pirilä, 1957). Subcutaneous implantation of Antabuse led to contact sensitization in two patients. Subsequent oral challenge with the hapten produced a flare-up reaction in one of the two patients (Lachapelle, 1975). A similar patient was described by Kiec-Swierczynska et al. (2000). Severe recall dermatitis of the penis was seen in a thiuram-sensitive patient after Antabuse treatment. He had been sensitized by the use of a rubber condom (Fisher, 1989). Antabuse also induces a systemic contact reaction by an entirely different mechanism. As Antabuse and its metabolites are strong metal-chelating substances, they can cause systemic contact reactions in nickel- and cobalt-sensitive patients via a pharmacological interaction in a dose-dependent manner (Kaaber et al., 1979, 1983; Veien, 1987c; Klein and Fowler, 1992).

Experimental oral challenge with 1 mg nickel before and during disulfiram treatment of a nickel-allergic patient showed greatly increased urinary nickel excretion during disulfiram treatment. A corresponding flare-up of dermatitis was seen (Hindsén et al., 1995).

The antitumor antibiotic mitomycin C is used for the treatment of superficial bladder cancer. Colver et al. (1990) demonstrated delayed-type hypersensitivity in 13 of 26 patients who

had received mitomycin installations by applying the allergen as a patch test (Colver et al., 1990). de Groot and Conemans (1991) reported six cases where intravesical administration of the drug resulted in a systemic contact dermatitis reaction including vesicular eczema of the hands and feet and dermatitis of the buttocks and genital area. A more widespread rash was eventually seen.

Calkin and Maibach (1993) reviewed delayed hypersensitivity to drugs and mentioned several patients who had positive patch tests to drugs and reactions to oral challenge with the same substances.

Other medications associated with systemic contact dermatitis are listed in Table 14.3.

TABLE 14.3
Medicaments That Have Caused Systemic Contact Dermatitis

Acetylsalicylic acid (Hindson, 1977)
Aminophylline
5-Aminosalicylic acid (Gallo and Parodi, 2002)
Amlexanox (Hayakawa et al., 1992)
Ampicillin
Antihistamines
Butylated hydroxy anisole (BHA), butylated hydroxy toluene (BHT)
Cinchocaine (Erdmann et al., 2001)
Clobazam (Machet et al., 1992)
Codeine (de Groot and Conemans, 1986)
Corticosteroids
Diclofenac (Alonso et al., 2000)
Dimethyl sulfoxide (Nishimura et al., 1988)
Ephedrine (Audicana et al., 1991)
Epsilon-Aminocaproic acid (Villarreal, 1999)
Erythromycin (Fernandez Redondo, 1994)
Estradiol (Gonçalo et al., 1999)
Gentamycin
Hydromorphone (de Cuyper and Goeteyn, 1992)
Hydroxyquinoline (Ekelund and Möller, 1969)
Immunoglobulins (Barbaud et al., 1999)
8-Methoxypsoralen (Ravenscroft et al., 2001)
Mitomycin C
Neomycin
Norfloxacin (Silvestre et al., 1998)
Nystatin (Lechner et al., 1987; Cooper et al., 1999)
Panthothenic acid (Hemmer et al., 1997)
Parabens (Kleinhans and Knoth, 1979)
Penicillin (Panhans-Gross et al., 1999)
Phenobarbitol (Pigatto et al., 1987)
Pristinamycine
Pseudoephedrine (Tomb et al., 1991; Sánchez et al., 2000)
Pyrazinobutanzone (Bris et al., 1992)
Resorcinol (Barbaud et al., 2001)
Streptomycin
Sulfonamides
Tetraaethylthiuram disulfide (Antabuse®)
Vitamin B_1 (Hjorth, 1958)
Vitamin C (Metz et al., 1980)

Note: Only references not mentioned in the text are given in the table.

14.5 NICKEL

Contact sensitivity to nickel is common, particularly among young females (Nielsen and Menné, 1992). Nickel-sensitive individuals seem to run an increased risk of developing hand eczema, particularly of the vesicular type (Wilkinson and Wilkinson, 1989). Christensen and Möller (1975) showed that oral intake of nickel induces a systemic contact dermatitis reaction in nickel-sensitive individuals. This observation led to intense research in the area of nickel dermatitis and systemic contact dermatitis (Menné and Maibach, 1991; Fowler, 1990).

Daily nickel intake varies from 100 to 800 μg (Biego et al., 1998; Ysart et al., 2000). The highest nickel content is found in vegetables, nuts, whole wheat or rye bread, shellfish, and cocoa. Nickel exposure from drinking water, air pollution, and cigarettes is usually negligible, although exceptions occur (Grandjean et al., 1989).

Certain makes of electric kettles and coffee machines and some glazed tea mugs may release significant amounts of nickel (Berg et al., 2000; Ajmal et al., 1997). Stainless steel cooking utensils contribute little to total nickel intake (Flint and Packirisamy 1995). Intravenous fluids may be contaminated with 100–200 μg Ni/L (Sunderman, 1983).

Only 1–10% of ingested nickel is absorbed. Nickel absorption varies greatly. Ingestion of 12 μg Ni/kg 1 h prior to eating a 1400 kJ portion of scrambled eggs gave a 13-fold higher serum concentration of nickel compared with the simultaneous ingestion of nickel and scrambled eggs (Nielsen, 1999). Both fecal and urinary nickel excretion can be used as parameters of systemic nickel exposure. The nickel concentration in sweat is high, ranging from 7 to 270 μg Ni/L (Grandjean et al., 1989; Hohnadel et al., 1973; Christensen et al., 1979).

Christensen and Möller (1975) challenged 12 nickel-sensitive female patients with an oral dose of 5.6 mg nickel given as nickel sulfate. Nine of the patients developed flares of the dermatitis with crops of vesicles on the hands. The reaction appeared within 2–16 h after ingestion. This observation has been confirmed (Table 14.4), and there is a marked dose–response relationship. Only a few nickel-sensitive patients react to oral doses of less than 1.25 mg of nickel, while most react to doses of 5.6 mg. A positive challenge test includes one or more of the previously described symptoms.

The flares seen at former nickel patch-test sites are also dose-dependent (Jensen et al., 2003) and are correlated to the intensity of the previous patch-test reaction and to the length of time since patch testing (Hindsén, 2001).

It is interesting that a previously seen statistical association between hand eczema and nickel sensitivity was no longer present 7 years after a ban on nickel content in items intended for close contact with the skin was introduced in Denmark in 1991 (Nielsen et al., 2002).

Hindsén et al. (1994) suggested that atopics absorb nickel more readily than nonatopics and that systemic nickel dermatitis should be looked for in atopics with nickel allergy.

There was rapid elimination of nickel in the urine after i.m. injection of nickel in hamsters, while elimination after

TABLE 14.4
Challenge Studies in Nickel-Sensitive Patients with an Oral Dose of Nickel Given as the Sulfate

Author	Type of Study	Allergen Dose (Elementary Nickel) (mg)	Duration of Dosing	Response Frequency
Christensen and Möller (1975)	Double blind	5.6	Single exposure	9/12
Kaaber et al. (1978)	Double blind	2.5	Single exposure	17/28
Kaaber et al. (1979)	Double blind	0.6	Single exposure	1/11
		1.2	Single exposure	1/11
		2.5	Single exposure	9/11
Veien and Kaaber et al. (1979)	Open	4.0	Single exposure	4/7
Jordan and King (1979)	Double blind	0.5	Two repeated days	1/10
Cronin et al. (1980)	Open	0.6	Single exposure	1/5
		1.25	Single exposure	4/5
		2.50	Single exposure	5/5
Burrows et al. (1981)	Double blind	2.0	Two repeated days	9/22
		4.0	Two repeated days	8/22
Goitre et al. (1981)	Open	4.4	Single exposure	2/2
Percegueiro and Brandao (1982)	Single blind	2.8	Repeated dose	34/43
		5.6		
Sertoli et al. (1985)	Open	2.2	Single exposure	13/20
Gawkrodger et al. (1986)	Double blind	0.4	Two repeated days	5/10
		2.5	Two repeated days	5/10
		5.6	Single dose	6/6
Veien et al. (1987a)	Double blind	2.5	Single exposure	55/131
Santucci et al. (1988)	Open	2.2	Single exposure	18/25
Hindsén et al. (2001)	Double blind	1.0		2/10
		3.0		9/9

cutaneous application of nickel was slow. Keratinocytes retained nickel much longer than did fibroblasts (Lacy et al., 1996).

The clinical implication of these findings is uncertain (Burrows, 1992; Möller, 1993). The nickel doses used in the challenge studies often exceed the amount of nickel in a normal daily diet. In experimental studies, we have often observed flare-up reactions at sites of previously positive nickel patch tests. This phenomenon has not been observed in clinical practice. After oral challenge with 0.6–5.6 mg nickel typically given as nickel sulfate, a nonphysiologically high concentration of urinary nickel was observed on the days following the challenge (20–200 μg Ni/L). In two studies (Menné and Thorboe, 1976; de Yongh et al., 1978) involving a small number of patients, higher nickel excretion in the urine tended to be related to active hand dermatitis, but the urinary nickel levels were much lower than the concentrations measured on the days following oral nickel challenge.

These observations do not exclude the possibility that systemic exposure to nickel is important for the chronicity of hand eczema related to nickel sensitivity. Undoubtedly, the daily nickel intake will sometimes exceed 0.6 mg, and two of five patients reacted to this dose in a study carried out by Cronin et al. (1980). A rather unpleasant diet with a high nickel content has been shown to increase the activity of chronic nickel dermatitis (Nielsen et al., 1990). A diet with low nickel content may diminish the activity of hand eczema in some nickel-sensitive patients (Veien et al., 1993b), and a flare of hand eczema has been seen in patients who abandoned such a diet (Veien et al., 1985a).

Dietary intervention controlled the dermatitis of 44 of 112 nickel-sensitive patients, and all except one patient who responded to the diet reacted to a placebo-controlled oral challenge with 2.23–4.47 mg nickel. The clinical manifestations were pruritic dermatoses, atopic dermatitis, and urticaria (Antico and Soana, 1999).

The inhalation of nickel while working in an electroplating plant caused a nickel-sensitive man to develop a widespread rash that cleared after disulfiram treatment and a low-nickel diet (Candura et al., 2001).

A nickel-sensitive woman who took two ampoules Oligosol® per day or the equivalent of a daily intake of 145.2 μg nickel/day had generalized dermatitis that cleared when Oligosol was discontinued (el Sayed et al., 1996). A study by Christensen et al. (1999) showed that Danish nickel-sensitive patients had lower serum nickel than controls. This appeared to be because the diet of the former had a lower nickel content.

If nickel is given intravenously to nickel-sensitive patients, 1–3 μg can elicit a severe systemic contact dermatitis reaction. This has been observed in patients treated with intravenous

infusions through cannulas that released traces of nickel and in patients treated with hemodialysis (Stoddard, 1960; Smeenk and Teunissen, 1977; Olerud et al., 1984; Raison-Peyron et al., 2005). This difference between oral and intravenous challenge doses indicates that very small variations in the amount of nickel in the skin may cause flares of dermatitis.

Nickel binds to a variety of naturally occurring proteins and amino acids (Nieboer et al., 1984). Thus, flares of dermatitis in nickel-sensitive patients may not always be caused by increased oral exposure to nickel but could be elicited by metabolic and pharmacological reactions. Interaction between nickel and zinc formed the basis for an open treatment trial in which 15 nickel-sensitive patients participated. The dermatitis of most of the patients for whom this assay was carried out improved or cleared, and their urinary nickel excretion was reduced (Santucci et al., 1999).

There may be cross-reactivity between nickel and palladium. Hindsén et al. (2005) challenged nickel- and palladium-sensitive patients with an oral dose of nickel in a placebo-controlled trial. More flare-up reactions were seen at sites of previously positive patch test to nickel and palladium following oral challenge with nickel than after ingestion of the placebo. This difference was statistically significant. Reactions at cobalt patch-test sites were also seen after oral challenge with nickel, but these were less frequent.

14.6 CHROMIUM, COBALT, AND OTHER METALS

Sidi and Melki (1954) suggested that oral dichromate ingestion in chromate-sensitive patients might be of importance for the chronicity of their dermatitis. This hypothesis has been tested in the studies listed in Table 14.5. Fregert (1965) challenged five chromate-sensitive patients with 0.05 mg chromium given as potassium dichromate. Within 2 h they developed severe vesiculation of the palms. One of the patients experienced acute exacerbation of generalized dermatitis. Schleiff (1968) observed flares of chromate dermatitis in 20 patients challenged with 1–10 mg potassium dichromate contained in a homeopathic drug. Some of the patients also experienced flares in previously positive dichromate patch-test sites.

Kaaber and Veien (1977) studied the significance of the oral intake of dichromate by chromate-sensitive patients in a double-blind study. Thirty-one patients were challenged orally with 2.5 mg chromium given as potassium dichromate and a placebo tablet. Nine of the eleven patients with vesicular hand eczema reacted with a flare of dermatitis within 1 or 2 days but did not react to the placebo. Three patients experienced vomiting, abdominal pain, and transient diarrhea after the chromate challenge, but not after challenge with the placebo.

A systemic contact dermatitis reaction to chromium has been seen after inhalation of welding fumes containing chromium (Shelley, 1964), after the ingestion of a homeopathic drug (van Ulsen et al., 1988), and after a nutritional supplement with chromium picolate (Fowler, 2000).

Compared to chromium and nickel, cobalt is well absorbed from the gastrointestinal tract. This makes cobalt-sensitive individuals candidates for further study of the possible existence of systemic contact dermatitis caused by this metal (Veien et al., 1987a). In a double-blind study, six of nine patients with positive patch tests to cobalt reacted to oral challenge with 1 mg cobalt given as 4.75 mg cobalt chloride (Veien et al., 1995). Most of the patients had recurrent vesicular hand dermatitis. Glendenning (1971) observed a 49-year-old housewife with persistent eczema of the palms and isolated cobalt allergy. After the removal of metal dentures made of a cobalt–chromium alloy (Vitallium), the dermatitis cleared. The patient had not had symptoms of stomatitis. After removal of the prostheses, she noticed a return of her appetite, the loss of which had been a definite symptom during the entire disease period.

Flare of cobalt dermatitis has been seen as a recall phenomenon in chronic alcoholics treated with tetraethylthiuram disulfide (Menné, 1985).

Systemically aggravated contact dermatitis has been caused by aluminium in toothpaste in children who have been sensitized to aluminium in vaccines (Veien et al., 1993a).

There have been several reports of widespread exanthema or multiforme-like erythema in patients with positive patch tests to mercury compounds (Nakayama et al., 1984). Vena et al. (1994) described nine such patients, seven of whom also had systemic symptoms such as malaise, pyrexia, and leukocytosis. The sensitization was induced by an antiparasitic

TABLE 14.5
Challenge Studies in Chromate-Sensitive Patients with an Oral Dose of Chromium Given as Potassium Dichromate

Author	Type of Study	Allergen Dose (Given as the Metal Chromium) (mg)	Duration of Dosing	Response Frequency
Fregert (1965)	Open	0.05	Single exposure	5/5
Scheiff (1968)	Open	1–10	Single exposure	20/20
Kaaber and Veien (1977)	Double blind	2.5	Single exposure	11/31
Goitre et al. (1982)	Open	7.1–14.2	Repeated exposure	1/1
Veien et al. (1994b)	Double blind	2.5	Single exposure	17/30

Note: 11 patients with pompholyx.

powder that was thought to cause systemic contact dermatitis after inhalation. Mercury in homeopathic medicine caused baboon syndrome in a 5-year-old girl (Audicana et al., 2001). Another route of systemic exposure is via dental treatment following the drilling of amalgam fillings. Following such treatment, a widespread maculopapular rash was seen in one mercury-sensitive patient (Aberer, 1993), two patients developed nummular dermatitis (Adachi et al., 2000), while another had flexural dermatitis (White and Smith, 1984) and one also had a flare of dermatitis at the site of a 4-week-old patch test to mercury (Veien, 1990). Flexural dermatitis is another manifestation of systemic dermatitis in mercury-sensitive patients. A baboon-like syndrome has also been seen (Pambor and Timmel, 1989; Faria and de Freitas, 1992; Zimmer et al., 1997).

A careful study of the concentration of mercury in saliva, feces, blood, plasma, and urine showed increased levels of mercury in saliva, blood, and feces during the first week after the removal of amalgam fillings. After removal of all the amalgam fillings, plasma Hg concentrations fell to 40% of the pretreatment level (Ekstrand et al., 1998).

Systemic contact dermatitis from implanted metals is rare with the currently employed technology within orthopedic surgery.

Case reports indicate that systemic contact dermatitis may still occur in a sensitized patient after the insertion of a metal prosthesis. Giménez-Arnau et al. (2000) reported widespread dermatitis in a nickel- and cobalt-sensitive woman whose aortic aneurism was repaired with a stent containing nickel and titanium.

A nickel-sensitive man developed vesicular hand dermatitis after his ankle fracture was repaired with plates containing 14% nickel. The dermatitis improved after the plate was removed (Kanerva and Förström, 2001).

Orthodontic appliances have been seen to cause urticaria and dermatitis in nickel-sensitive persons (de Silva and Doherty, 2000; Kerosuo and Kanerva, 1997; Fernández-Redondo et al., 1998). In some nickel-sensitive patients, the diagnosis has required oral challenge with the metals nickel, cobalt, and chromium (Veien et al., 1994a).

Gold has become a common contact allergen in several centers. Möller et al. (1996) challenged 20 gold-sensitive patients with sodium thiomalate or placebo. One of ten who received the active compound experienced flare-up of a previous contact dermatitis site. All 10 patients experienced a flare-up of their previous gold patch-test sites, and several patients had toxicoderma-like symptoms. In a later study, Möller et al. (1999b) saw a flare-up of previously positive gold patch-test sites and transient fever in five of the five gold-sensitive patients. Russell et al. (1997) reported three patients who developed lichenoid dermatitis after drinking liquor containing gold.

14.7 OTHER CONTACT ALLERGENS

Kligman (1958a) attempted to hyposensitize persons with *Rhus* dermatitis by giving increasing amounts of the allergen in oral doses. Half of the moderately to severely sensitive patients experienced either pruritus or a rash; 10% of the patients experienced flares of their dermatitis at sites of previously healed contact dermatitis. Flares of vesicular hand eczema and erythema multiforme were rare. Pruritus ani occurred in 10% of the highly sensitive individuals. Severe systemic contact dermatitis was described in *Rhus*-sensitive patients who had eaten cashew nuts (Ratner et al., 1974). The allergen in cashew nut shells cross-reacts with the poison ivy–allergen, which explains the reactions (Kligman, 1958b).

Sporadic cases of cashew nut dermatitis caused by the presence of shell fragments among edible nuts have been described. A case of perianal dermatitis occurred after the ingestion of cashew nut butter (Rosen and Fordice, 1994). A baboon-like eruption occurred 36 h after the ingestion of a pesto sauce containing cashew nuts (Hamilton and Zug, 1998).

The lacquer tree contains antigens related to those found in poison ivy and cashew nuts. Thirty-one patients with systemic contact dermatitis were seen following the ingestion of lacquer. A widespread erythematous, maculopapular eruption was the most common clinical symptom. Some patients experienced abdominal pain, nausea, vomiting, and chills (Park et al., 2000).

Systemic contact dermatitis has been seen in patients sensitive to balsam of Peru, which contains naturally occurring flavors. The perfume mixture may be a better indicator of sensitivity to spices than balsam of Peru (van der Akker et al., 1990). Hjorth (1965) observed systemic contact dermatitis in balsam of Peru–sensitive patients who had eaten flavored icecream and orange marmelade. Veien et al. (1985b) challenged 17 patients sensitive to balsam of Peru with an oral dose of 1 g of balsam of Peru. Ten patients reacted to balsam of Peru and one to a placebo.

Hausen (2001a) reviewed 102 patients sensitive to balsam of Peru. Ninety-three reacted to one or more of 19 constituents. Eight who had reactions to coniferyl benzoate and benzyl alchohol had systemic contact dermatitis. Three of these patients had hand eczema and three had widespread dermatitis.

Based on questionnaires mailed to the patients 1–2 years after the initiation of diet treatment, Veien et al. (1996a) reviewed 46 balsam-sensitive patients who had been asked to reduce their dietary intake of balsams. Sixteen of 22 (73%) who had reacted to 1 g balsam of Peru in a placebo-controlled oral challenge had benefit from a low-balsam diet compared to 3 of 10 (30%) who had shown no reaction to the oral challenge. Nine of fourteen (64%) who were placed on a low-balsam diet, but who were not challenged orally, benefited from a low-balsam diet.

Salam and Fowler (2001) studied 71 perfume and balsam-sensitive patients retrospectively. The dermatitis of 21 of 45 patients who followed a low-balsam diet improved or cleared. The most common sites of dermatitis were the hands, face, and anogenital region. The most commonly implicated foods were tomato, citrus, and spices.

Niinimäki (1995) challenged 22 patients orally with balsam of Peru in a placebo-controlled study. Eight patients reacted to balsam of Peru but not to the placebo, while four

reacted to both balsam of Peru and the placebo or only to the placebo. Aggravation of vesicular hand eczema was the most common clinical response. Similarly, Niinimäki (1984) challenged 71 patients sensitive to balsam of Peru with spices. Seven had positive reactions to the challenge. Most had vesicular hand eczema.

A 56-year-old woman had dermatitis on the fingers of both hands, and patch testing showed a + reaction to balsam of Peru and a +++ reaction to coniferyl benzoate. Her hand eczema cleared after she stopped smoking and drinking 3 L of Coca-Cola® per day (Hausen, 2001b).

The dermatitis of two balsam of Peru–patients cleared after a reduction in the intake of plant extracts (le Sellin, 1998), and the hand eczema of a patient sensitive to balsam of Peru cleared after a low-balsam diet was followed. The latter diagnosis was confirmed by oral challenge with balsam of Peru (Pfutzner et al., 2003).

A patient sensitive to balsam of Peru and to rosin experienced a flare of hand eczema and widespread dermatitis after dental work involving the filling of a root canal with rosin (Bruze, 1994).

Dooms-Goossens et al. (1990) described systemic contact dermatitis caused by the ingestion of spices in a patient with a positive patch test to nutmeg and in two patients sensitive to plants of the composita family after the ingestion of laurel. Sesquiterpene lactones found in compositae caused systemic contact dermatitis in a patient following the ingestion of lettuce (Oliwiecki et al., 1991).

Goldenrod in an oral medication (Urodyn®) caused systemic contact dermatitis in a 52-year-old man (Schätzle et al., 1998).

German chamomile tea caused a widespread eruption and anal pruritus in a 26-year-old woman who was sensitive to sesquiterpene lactone (Rodríguez-Serna et al., 1998) and caused recurrent facial dermatitis in another patient (Rycroft, 2003).

Inhalation of the allergen costus resinoid caused a baboon-like eruption in a sesquiterpene lactone-sensitive woman (le Coz and Lepoittevin, 2001).

A 45-year-old man developed widespread dermatitis after the ingestion of tea tree oil to which he had previously had a positive patch test (de Groot and Weyland, 1992).

Kava extract caused systemic contact dermatitis in one patient (Suss and Lehmann, 1996).

Garlic has been shown to cause systemic contact dermatitis with vesicular hand eczema as the clinical manifestation. The dermatitis could be reproduced by placebo-controlled oral challenge (Burden et al., 1994). Ingestion of garlic has also caused systemic contact dermatitis in the elbow flexures and periorbitally (Pereira et al., 2002).

Cutaneous reactions following the ingestion of alcoholic beverages were reviewed by Ophaswongse and Maibach (1994). Both immediate- and delayed-type hypersensitivity reactions causing systemic contact dermatitis were described. One patient became sensitized to ethanol in an estrogen transcutaneous delivery system. She developed widespread exanthema after the ingestion of alcoholic beverages (Grebe et al., 1993).

The antioxidant butylated hydroxyanisole, which is used both in cosmetics and in foods, can cause systemic contact dermatitis (Roed-Petersen and Hjorth, 1976), as can substances as diverse as formaldehyde (Bahmer and Koch, 1994) and ethyl ethoxymethylene cyanoacetate (Hsu et al., 1992).

Preservatives such as sorbic acid have caused systemic contact dermatitis presenting clinically as hand eczema (Raison-Peyron et al., 2000; Dejobert et al., 2001).

Parabens have been suspected as the cause of systemic contact dermatitis. However, only 2 of 14 paraben-sensitive patients experienced flares of their dermatitis after placebo-controlled oral challenge with 200 mg methyl and propyl parahydroxybenzoate. Both patients who reacted to the challenge had vesicular hand eczema (Veien et al., 1996b).

14.8 RISK ASSESSMENT-ORIENTED STUDIES

While the risk of systemic contact dermatitis from drugs can be assessed, it is more difficult to carry out similar studies on ubiquitous contact allergens such as metals and naturally occurring flavors. In spite of intensive research on the significance of orally ingested nickel in nickel-sensitive individuals, we are unable to give firm advice concerning the oral dose that would represent a hazard for the wide range of nickel-sensitive individuals. Many variables, such as the route of administration, bioavailability, individual sensitivity to nickel, interaction with naturally occurring amino acids, and interaction with drugs must be considered. A number of as yet unknown factors could influence nickel metabolism. Furthermore, immunological reactivity to nickel can change with time and can be influenced by sex hormones and the development of tolerance. It is important to recognize that this area of research is extremely complex and that much well-controlled research is still needed.

Jensen et al. (2006) performed a modified meta-analysis of the theoretical risk of systemic contact dermatitis after the oral administration of nickel in nickel-sensitive patients. The conclusion was that only a minority of nickel-sensitive patients is at risk of systemic contact dermatitis after the ingestion of nickel in food.

With regard to medicaments, it is possible to perform well-controlled oral challenge studies in sensitized individuals. The beta-adrenergic blocking agent alprenolol is a potent contact sensitizer. Ekenvall and Forsbeck (1978) identified 14 workers employed in the pharmaceutical industry who were contact-sensitized to this compound. Oral challenge with a therapeutic dose (100 mg) led to a flare in one worker, who experienced pruritus and widespread dermatitis.

Merthiolate is a preservative widely used in sera and vaccines. Förström et al. (1980) investigated 45 merthiolate contact-sensitive persons to evaluate the risk of a single therapeutic dose of 0.5 mL of a 0.01% merthiolate solution given subcutaneously. Only 1 of the 45 patients developed a systemic contact dermatitis reaction. Aberer (1991) did not observe any reactions in a similar study involving 12 patients.

Maibach (1987) studied a group of patients who had discontinued the use of transdermal clonidine because of

dermatitis. Of 52 patients with positive patch tests to clonidine, 29 were challenged orally with a therapeutic dose of the substance. Only one patient reacted with a flare-up at the site of the original dermatitis.

Propylene glycol is used as a vehicle in topical medications and cosmetics and as a food additive. Propylene glycol is both a sensitizer and a primary irritant. Hannuksela and Förström (1978) challenged 10 contact-sensitized individuals with 2–15 mL propylene glycol. Eight reacted with exanthema 3–16 h after the ingestion.

14.9 DIAGNOSIS

Systemic contact dermatitis can occur in patients who are contact sensitized to haptens if these patients are then exposed systemically to the same hapten. The number of persons who will actually react to a systemic exposure depends on the dose administered and for nickel to the strength of the patch-test reaction and the time elapsed since patch testing (Hindsén, 2001). Break-down products like formaldehyde from aspartame may cause systemic contact dermatitis (Hill and Belsito, 2003).

According to the available literature, particularly from experimental nickel challenge studies and challenge studies with medicaments, the dose needed to produce such systemic contact dermatitis reactions is relatively large. The number of patients with systemic contact dermatitis seen in clinical practice is low compared to the number of patients with allergic and irritant contact dermatitis (Veien et al., 1987b). In spite of the fact that systemic contact dermatitis is relatively rare, it is important to identify this type of reaction to provide optimal management of the individual patient. The diagnosis rests upon patch testing and oral-challenge studies. Severe reactions are exceptional. To our knowledge, severe or lethal anaphylactic reactions have not occurred after accidental or experimental oral challenge of patients with allergic contact dermatitis.

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15 Allergic Contact Dermatitis

Francis N. Marzulli and Howard I. Maibach

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Contact dermatitis, an inflammatory skin disease characterized by itching, redness, and skin lesions, is caused by skin contact with either an irritant or an allergenic chemical. Acute irritant contact dermatitis arises on initial contact with an adequate concentration of a direct-acting cytotoxic chemical. Whereas, allergic contact dermatitis (ACD) usually arises following more than one skin contact (induction and elicitation) with an allergenic chemical. The skin response of ACD is delayed, immunologically mediated, and consists of varying degrees of erythema, edema, and vesiculation. In the Gell and Coombs system (1968), it is classified as a cell-mediated, tuberculin-like, Type IV allergy.

The best known example of ACD is the linear vesicular skin response that is often seen hours after contact with poison ivy, at which time itching is a prominent symptomatic feature.

Allergenic chemicals penetrate the skin as small molecules (usually $<$400 MW), and they are incompletely allergenic (haptens) until they bind to protein and form a complete allergen.

In ACD, the first significant exposure to a haptenic chemical activates the immune system (induction) and sensitizes the skin. After sensitization (which takes a few days to a few weeks), subsequent antigenic exposures result in the evocation of an altered ("allergic") skin response (elicitation), that is, one that is more pronounced than the original response. For sensitization to take place, the allergenic chemical must first penetrate the skin, so that it can reach and interact with key elements of the underlying immune system. A certain level of allergen entry must be achieved that represents a threshold for triggering the immune system. The threshold can be reached following a single skin exposure to a sufficiently high amount or concentration of allergenic chemical, or after contact with a large area of skin, or as a consequence of repeated skin applications.

Once the allergenic chemical has abrogated the horny barrier layer of skin and entered the viable layer of the epidermis, it makes contact and binds with Langerhans cells. These are dendritic cells that direct the allergen to a regional lymph node where interaction with T lymphocytes is followed by replication of sensitized T lymphocytes and expansion of the sensitized T-lymphocyte population, completing the induction phase of the sensitization process.

In the sensitized individual, the next contact with the allergenic chemical results in the elicitation of a hypersensitive skin response that is due to a reaction between circulating sensitized lymphocytes and allergen at the skin site where allergen has entered the living epidermis.

The term allergy was coined by von Pirquet in 1910; however, our present understanding of events of the sensitization process probably began to unfold after the patch test was developed by Jadassohn (1895). This test was intended to clinically duplicate contact allergy on a small scale by applying a suspect chemical to the skin of a sensitized patient (under occlusion) to confirm its allergenic potential.

The next important episode involved the passive transfer of immediate-type allergy by the Prausnitz-Kustner reaction (1921). This consisted of injecting blood serum from an allergic person into the skin of a normal person, making the injected site reactive to the injected allergen.

Landsteiner and Jacobs (1936) demonstrated that delayed-type reactions could be induced by intradermal injection of certain allergens.

Another important finding was that of Landsteiner and Chase (1942) that lymphocytes become sensitized during the development of ACD.

Other early historical events were reviewed in the 50th anniversary issue of the *Journal of Allergy and Clinical Immunology* (Allergy, 1979).

The development of predictive and diagnostic human and guinea pig tests for skin sensitization focused further attention on ACD (Schwartz, 1941), as did regulatory and legal requirements for evaluating drug and cosmetic safety (Draize, 1959).

Early in the study of ACD, humans were the primary investigative test species. Later, guinea pigs were added as the animal model of choice. More recently the mouse has been used extensively.

Concordance of developments in genetics and molecular biology, based on mouse studies, with the entry of the mouse as a test species for ACD potential led ultimately to the finding that cytokines play a role in both irritant dermatitis and ACD. A detailed interpretation of cellular and molecular events of ACD is given in Sauder and Pastore (1993).

Entrance of an irritant or allergenic chemical into the epidermis signals the release of a cascade of cytokines from

affected keratinocytes, suggesting a key role for keratinocytes in these inflammatory processes. It is not yet clear how cytokines differ qualitatively and quantitatively during ACD and irritant reactions, but this is likely to be an important area of future research.

While foundations for the overall picture of events of ACD appear secure, the future may unhinge some present interpretations of the details.

Keratinocytes comprise the main cellular composition of the human epidermis. They are involved in synthesis of various cytokines during both normal and abnormal cell functions. Cytokines are regulatory proteins that mediate cell communication, and include interleukins, growth factors, colony-stimulating factors, and interferons. When keratinocytes are damaged during contact with irritant or allergenic compounds, various inflammatory elements, including cytokines, adhesion molecules, and chemotactic factors, are released (Barker et al., 1991; Kupper, 1989). Current research interest in this area has sparked a continuously expanding literature.

A number of studies have been conducted to investigate the activation sequence following keratinocyte damage. In one such study (Willis et al., 1989), 10 healthy human volunteers were patch tested with six structurally unrelated irritants, and their skins were biopsied 48 h later and examined by light and electron microscopy. Keratinocytes were damaged in distinctly different patterns, and mononuclear cells were the predominant infiltration response.

It is virtually impossible to distinguish irritant dermatitis from ACD with precision, on gross and even microscopic inspection. Recently, Brasch et al. (1992) reported an attempt to find a distinction between the two dermatides. Topically applied sodium lauryl sulfate (SLS), an irritant chemical, was administered to seven sensitized subjects along with the allergenic chemical, on two separate test skin sites, to produce experimental irritant and allergic contact dermatitis on a small scale. Both skin sites responded similarly in clinical appearance, histology, and immunohistology. A large battery of monoclonal antibodies directed against numerous surface, intracellular, and nuclear antigens failed to uncover a difference between the irritant and the allergic sites by these immunostaining techniques.

The finding that cytokines are released during the ACD process opens the way to the ultimate development of a specific test to differentiate ACD from irritant dermatitis (Enk and Katz, 1992; Paludan and Thestrup-Pederson, 1992).

Some commonly encountered allergens that have been reported by members of the North American Contact Dermatitis Research Group during diagnostic studies of clinical patients include (Storrs et al., 1989) balsam of Peru, benzocaine, benzoyl peroxide, black rubber paraphenylenediamine mix, caine mix minus benzocaine, carba mix, cinnamic alcohol, cinnamic aldehyde, dibucaine, cyclomethycaine sulfate, epoxy resin, ethylenediamine dihydrochloride, eugenol, formaldehyde, hydroxycitronellal, imidazolidinyl urea, isoeugenol, lanolin alcohol, mercapto rubber mix, mercaptobenzothiazole, neomycin sulfate, nickel sulfate, oak moss, *p*-tertiary butylphenol formaldehyde resin, potassium dichromate, *p*-phenylene diamine, quaternium-15, rosin (colophony), tetracaine, thimerosol, and thiuram rubber mix.

Vehicle and preservative allergens include ammoniated mercury, benzophenone, 2-bromo-2-nitropropane 1, 3-diol (bronopol), captan, chloroacetamide, *p*-chloro-*m*-cresol, chloroxylenol, diazolidinyl urea, dichlorophene, DMDM hydantoin, Kathon CG, paraben mix, polythylene glycol, *o*-phenylphenol, propylene glycol, sorbic acid, and Tween 85.

Cosmetic injury reports to the Office of Cosmetics and Colors, FDA (1988–1993), are similar to preceding years when fragrances and preservatives were established as the most common sensitizers in cosmetics.

Among the newer preservative chemicals that may sensitize are methyldibromoglutaronitrile and phenoxyethanol (Euxyl K 400). Dibenzoyl methanes in addition to PABA (*para*-aminobenzoic acid) are among the newer sunscreens that are potential allergens (Brancaccio, 1992). Furthermore, glyceryl monothioglycolate is a sensitizer in hair products; nicotine is in transdermal therapeutic systems; and corticosteroids such as hydrocortisone are of increasing concern as sensitizers (Brancaccio, 1992).

Natural rubber latex, now widely used in surgical gloves and in condoms, is producing allergic reactions in epidemic proportions.

Additional useful information relating to the allergenic potential of specific chemicals is published in other scientific journals.

The Cosmetic Ingredient Review Committee of the Cosmetic, Toiletry and Fragrance Association has published 22 special issues of the *Journal of the American College of Toxicology* that contain information on skin sensitization and other safety data on cosmetic ingredients beginning in 1982.

The Research Institute for Fragrance Material has published sensitization data on chemicals that have been proposed for use as fragrance ingredients and are in *Food and Chemical Toxicology*.

Lachapelle (2003) provides an efficient premise on diagnostic skin testing. Maibach (2001) dermatotoxicologic assayz. Wahlberg (2003) summarizes common exposures to the more frequently patch tested chemicals.

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16 Mechanisms in Irritant and Allergic Contact Dermatitis

Iris S. Ale and Howard I. Maibach

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16.1 INTRODUCTION

Contact dermatitis is one of the most common skin diseases, with a great socio-economic impact (Uter et al., 1998). As the outermost barrier of the human body, the skin is the first to encounter chemical and physical agents from the environment. According to the pathophysiological mechanisms involved, two major types of contact dermatitis may be recognized, that is, irritant contact dermatitis (ICD) and allergic contact dermatitis (ACD).

The basis for a diagnosis of either ICD or ACD is mainly established by considering the morphology of the clinical lesions, assessing the relationship between the putative exposure and the time course of the dermatitis, as well as performing appropriate diagnostic patch testing. Differentiating between ICD and ACD is frequently difficult in the clinical setting. This represents a considerable predicament, in view of the high frequency of these entities and their impact on the patient's quality of life. Discriminating between allergens and irritants is also crucial in toxicological research and major efforts have been made to identify morphological, immunohistochemical, and immunological changes, allowing for a clear-cut distinction between the effects of allergens and irritants.

Such information will enhance our understanding of the molecular processes involved in ICD and ACD and may provide a mechanistic basis for designed refined *in vivo* and *in vitro* models to be applied in toxicological testing.

16.2 PATHOGENETIC MECHANISMS

Several authors have demonstrated that ACD and ICD may have clinical, histological, and immunohistochemical similarities (Lammintausta and Maibach, 1990; Brasch et al., 1992; Scheynius et al., 1984). However, the immunobiological mechanisms that underlie both types of response were thought to be fundamentally different.

ACD requires the activation of antigen specific–acquired immunity leading to the development of effector T cells, which mediate the skin inflammation. The cutaneous inflammatory response in ACD is dependent on the activation and clonal expansion of specific allergen-responsive T lymphocytes. In contrast, ICD has been defined as a local inflammatory reaction, following a single or repeated exposure to an irritant, which is an agent producing a direct toxic insult to the cutaneous cells (Mathias and Maibach, 1978). Irritant damage to the skin induces abnormalities of epidermal proliferation and differentiation and activation of the skin innate immunity. Epidermal cells injured by irritants release eicosanoids, cytokines, and growth-enhancing factors, which are potent chemoattractants for leukocytes and may induce T-cell activation via antigen-independent pathways (Barker et al., 1991; Nickoloff and Naidu, 1994; McKenzie and Sauder, 1990). The immune cascade is then activated independently of the antigen presentation pathway, by the induction of proinflammatory mediators that directly recruit and activate T lymphocytes, without the induction of antigen-specific memory T cells (Baadsgaard and Wang, 1991; Hunziker et al., 1992). Following T-cell activation and lymphokine release, the cellular events and inflammatory response in ACD and ICD seem to be comparable (Ulfgren et al., 2000; Effendy et al., 2000; Brand et al., 1997; Gawkrodger et al., 1986; Brasch et al., 1992).

Therefore, the distinction between ICD and ACD becomes progressively blurred, and it is now apparent that allergic and irritant contact reactions have at least partially overlapping pathophysiology and share common effector pathways (Brand et al., 1996; Effendy et al. 2000; Mohamadzadeh et al., 1994).

Moreover, many chemicals that are capable of behaving as contact allergens also have irritant properties, frequently disregarded because their allergenic potential dominates their toxicity profile. Indeed, it is believed that irritancy promotes allergic sensitization to some degree, even if the mechanisms underlying this interaction are only partially known. The "danger signal" hypothesis (Matzinger, 1998) suggests that the immune responses can be augmented by danger signals released by tissues undergoing stress, damage, or abnormal death. In this context, an antigenic signal will produce sensitization only in the presence of a danger signal; in its absence tolerance will occur. Irritancy may represent a danger signal for the immune system as direct toxic damage of dendritic cells (DC) or other cells (principally keratinocytes) will trigger cytokine release predisposing to allergic sensitization (Gallucci and Matzinger 2001; McFadden and Basketter, 2000). In fact, as most contact allergens have irritant properties, both the antigenic and danger signals may arise from the hapten (Smith et al., 2002).

The majority of haptens bear lipophilic residues, which enable them to cross through the stratum corneum (SC) barrier, and electrophilic residues, which are responsible for the covalent bonds to the nucleophilic residues of cutaneous proteins (Lepoittevin and Leblond, 1997). T lymphocytes recognize haptens as structural entities bound covalently to, or by complexation to, peptides anchored in the grooves of major histocompatibility (MHC) class I and class II molecules. The antigen able to activate hapten-specific T cells is then a haptenated peptide (Martin et al., 1992).

The pathophysiology of contact sensitization (CS) has been classically divided into two distinct phases, that is, the sensitization phase and the elicitation phase. The sensitization phase, also referred to as afferent phase or induction phase of CS, was believed to occur at the first significant contact of skin with the sensitizing substance, which leads to the generation of allergen-specific T cells. The hypothesis of the first contact of a chemical being sensitizing for the host may be accurate for strong allergens; however, ACD to moderate or weak allergens almost never occurs after the first contact but may take years of permanent skin exposure to develop.

The haptens capable to induce CS are low molecular weight chemicals that are not immunogenic by themselves, and need to bind to epidermal proteins that can act as carrier molecules. The ability of a hapten to induce sensitization relies on two distinct properties. Through their proinflammatory properties, haptens activate the skin innate immunity and deliver signals responsible for inducing the maturation and migration of cutaneous DC. Through their binding to amino acid residues they modify self-proteins and induce the expression in the skin of new antigenic determinants. Haptens are loaded by DC and are expressed as haptenated peptides in the groove of MHC class I and class II molecules at the cell surface. Hapten-bearing DC migrate from the skin to the regional lymph node where specific CD8+ and CD4+ T lymphocytes are primed in the paracortical area. T cells proliferate and emigrate out of the lymph nodes to the blood where they recirculate between the lymphoid organs and the skin.

In the elicitation phase, also known as efferent or challenge phase of CS, the challenge of sensitized individuals with the same hapten leads to the apparition of ACD/CS in 24/72 h. Haptens diffuse in the skin and are uptaken by skin cells, which express MHC I and II/haptenated peptide complexes. Specific T lymphocytes are activated in the dermis and the epidermis, and trigger the inflammatory process responsible for the cutaneous lesions. Recent studies have demonstrated that CD8+ cytotoxic T lymphocytes are the main effector cells of CHS to strong haptens and that they are recruited early after challenge before the massive infiltration of leucocytes, which contain the downregulatory cells of CHS, found in the CD4+ T-cell subset.

16.2.1 Cytokine Profiles

Cytokines, a family of inducible glycoproteins, play a fundamental role in triggering and developing the inflammatory processes occurring in the skin. Therefore, several studies have investigated the cytokine expression in allergic and irritant reactions, in an effort to provide insight about the underlying pathogenic mechanisms operating in both types of inflammatory responses. Until now, the results have been contradictory and have not provided unambiguous differentiation (Table 16.1).

After skin contact, irritants such as sodium lauryl sulfate (SLS) impair the SC barrier (De Jongh et al., 2006; Fartasch, 1997; Willis et al., 1989) and produce a direct toxic effect on the keratinocytes. In response to these effects, preformed cytokine interleukin-1α (IL-1α) is released from the SC and keratinocytes as the first step in the inflammatory cascade. IL-1α stimulates other keratinocytes and fibroblasts to produce and release more IL-1α, as well as other proinflammatory cytokines such as IL-1β, IL-6, IL-8, and tumor necrosis factor-α (TNF-α), which may play a role in the migration and activation of inflammatory cells (Boxman et al., 1996). The triggered cytokine cascade results in the inflammatory response induced by irritants. Hoefakker et al. (1995) attempted to elucidate whether there was a difference in the cytokine profiles in allergic and irritant patch-test reactions, especially at the same time of final reading in diagnostic patch testing. Local cytokine profiles in skin biopsies from allergic reactions to epoxi resin (1%) and formaldehyde (1%), and irritant reactions to SLS (10%) and formaldehyde (8%) were determined by *in vivo* immunohistochemistry. The allergic and irritant patch-test reactions showed similar levels of expression of the Th1 cytokines IL-2 and interferon-γ (IFN-γ) in the dermis, confirmed by probe-based detection of IL-2 mRNA and IFN-γ mRNA.

In cultured human keratinocytes, different irritants, namely SLS, phenol, and croton oil, as well as the allergen dinitrofluorobenzene (DNFB) induced the production and intracellular accumulation of IL-8 (Wilmer et al., 1994). Similarly, the expression of IL-8 gene by human keratinocytes was significantly increased by SLS and the allergens DNFB and 3-*n*-pentadecylcatechol (Mohamadzadeh et al., 1994). Ulfgren et al. (2000) observed that the cytokine

TABLE 16.1
Cytokines, Chemokines, and Growth Factors Profiles in ICD and ACD (Human Studies)

Expression	ICD	ACD	Reference
IL-1α, β	Upregulated (or not altered)	Upregulated	Hoefakker et al. (1995), Ulfgren et al. (2000), Brand et al. (1996), Enk et al. (1993b), Hunziker et al. (1992), Oxholm et al. (1991)
IL-2	Upregulated (or not altered)	Upregulated	Hoefakker et al. (1995), Ulfgren et al. (2000), Ryan and Gerberick (1999), Hunziker et al. (1992)
IL-4	Not altered (or increased)	Upregulated at 24 h	Ryan and Gerberick (1999)
IL-6	Upregulated	Upregulated	Ulfgren et al. (2000), Oxholm et al. (1991), Hunziker et al. (1992), Hoefakker et al. (1995)
IL-8	Upregulated	Upregulated	Wilmer et al. (1994), Mohamadzadeh et al. (1994)
IL-10	Not altered (or increased)		Ulfgren et al. (2000), Brand et al. (1997), Ryan and Gerberick (1999)
TNF-α	Upregulated (or not altered)	Upregulated	Hoefakker et al. (1995), Hunziker et al. (1992), Oxholm et al. (1991)
IFN-γ	Upregulated	Upregulated	Pichowski et al. (2000), Hoefakker et al. (1995)
IP-10, IP-9	Not altered	Upregulated	Flier et al. (1999)
MIF	Not altered	Upregulated	Flier et al. (1999)
GM-CSF	Upregulated	Upregulated	Hunziker et al. (1992)

profile in irritant reactions to SLS and contact allergic skin reactions to nickel 6 h after challenge was similar. At 72 h, the dermal cells expressed IL-1α, IL-1β, IL-2, IL-4, IL-6, and IL-10 in both types of inflammatory reactions. However, two differences were observed. Staining for the IL-1 receptor antagonist was more prominent in the dermis at the late stages of the allergic reaction, and the inflammatory mononuclear infiltrate showed a more prominent IFN-γ staining in the irritant reactions. Pichowski et al. (2000) studied the mRNA expression for IL-1β in blood-derived dendritic cells, cultured in the presence of DNFB, SLS, or vehicle. This cytokine plays a major role in the induction phase of ACD (Enk et al., 1993b) and was shown to upregulate MHC class II molecule expression on Langerhans cells (LC) *in situ* to produce recruitment of inflammatory cells and to induce adhesion molecules related to leukocyte–endothelial adhesion. A two- to threefold increase in IL-1β mRNA was observed in cells derived from three of eight DNFB-treated donors, whereas SLS treatment did not induce IL-1β mRNA expression in the cells of any of the donors investigated. In contrast, Brand et al. (1996) demonstrated that the protein levels of IL-1β in human skin lymph increased in the course of both irritant and allergic contact dermatitis and therefore do not allow to discriminate between them.

Using an *in situ* hybridization technique, Flier et al. (1999) detected mRNA expression for the chemokine IFN-γ-inducible-protein-10 (IP-10), as well as the related CXC chemokine receptor-3 (CXCR3) activating chemokines, macrophage migration inhibitory factor (Mif), and IP-9 in contact allergic reactions, but not in SLS-induced irritant reactions. Additionally, up to 50% of the infiltrating cells in ACD expressed CXCR3, the cognate receptor for IP-10, Mif, and IP-9, which is nearly exclusively expressed on activated T cells. In contrast, CXCR3 expression was found in only 20% of irritant reactions. The differential expression of IP-10 in human ICD and ACD is consistent with the results of previous studies in mice (Enk and Katz, 1992; Enk et al., 1993a) and suggests that this chemokine intervene in the generation of the inflammatory infiltrate in ACD, but not in SLS-induced irritant reactions. In addition, intercellular adhesion molecule 1 (ICAM-1) expression by keratinocytes was only found in allergic reactions correlating with chemokine expression (Flier et al., 1999). Similarly, Verheyen et al. (1995) and Vestergaard et al. (1999) reported that ICAM-1 expression in keratinocytes can be found in allergic patch test reactions but only rarely occurred in irritant reactions induced by SLS or croton oil. These results support the conception that ICAM-1 plays a role in the specific immune response by facilitating the antigen presentation or lymphocytic infiltration. However, upregulation of ICAM-1 expression by keratinocytes, in correlation with expression of lymphocyte function antigen-1-positive leukocytes, was also observed in SLS-induced reactions (Willis et al., 1991), indicating that ICAM-1 may play a role in irritant reactions. Similarly, Verheyen et al. (1995) reported that ICAM-1 expression by endothelial cells and also mononuclear cells could be induced in both irritant and allergic reactions.

Brand et al. (1997) observed that the IL-10 levels in lymph derived from irritant reactions and primary sensitization of ACD were similar to those obtained from normal skin, remaining below 4.4 pg/mL. In contrast, the IL-10 levels increased manifold, both in the primary allergic reaction (928.5 pg/mL) and the elicitation of ACD (124 pg/mL). In addition, the IL-10 mRNA signal was markedly stronger in lymph and epidermal blister cells from the elicitation reactions as compared to the signal in lymph cells derived from normal skin and from the primary sensitization of allergic reactions. Similarly, Ryan and Gerberick (1999) observed stronger mRNA expression of IL-10, IL-4, and IL-2 in allergic patch-test reactions to poison ivy compared to irritant reactions induced by SLS.

The number of infiltrating cells was larger in biopsies from allergic reactions induced by nickel sulfate than in irritant reactions induced by SLS. However, at the single-cell level, the expression of VLA (very late antigens), lymphocyte function-associated antigen-1 (LFA-1), CD44, and intercellular adhesion molecule-1 (ICAM-1) was similar in both groups. The endothelial cells in allergic reactions showed a stronger expression of vascular cell adhesion molecule-1 (VCAM-1), endothelial leukocyte adhesion molecule-1 (ELAM-1), and ICAM-1 compared to irritant reactions (Wahbi et al., 1996).

In summary, even if cytokines play a fundamental role in the pathogenic mechanisms of inflammatory skin diseases, present knowledge of the complex interactions of cytokines and cellular targets does not allow for identification of a specific "fingerprint" pattern of cytokine production that clearly distinguishes allergic from irritant reactions.

16.3 HISTOLOGY AND IMMUNOHISTOCHEMISTRY

Cellular changes that take place during the development of ACD and ICD, including modifications in the morphological appearance, proliferative state, and cell kinetics, have been evaluated in an attempt to discriminate between both disorders.

ICD shows much greater histological pleomorphism than ACD (Table 16.2). Irritants produce a wide range of histopathological changes as a consequence of their different mechanism of action and chemical interaction with the skin components. Lesions will also vary according to concentration of the irritant, type and duration of the exposure, and individual reactivity of the skin (Lachapelle, 1995; Willis et al., 1992, 1988; Patrick et al., 1985). Acute allergic reactions are characterized by dermal inflammatory infiltrates around the dilated venules of the superficial plexus, edema, and spongiosis. The inflammatory infiltrate in the epidermis adopts a focal distribution (Avnstorp et al., 1987) and constitutes microvesicles. If the process evolves more slowly, the spongiosis propels the epidermis to become hyperplastic. Subacute ACD lesions of ACD become less spongiotic and more psoriasiform. In chronic lesions of ACD there is almost no spongiosis. Rubbing and scratching will cause lichenification with thickening of the epidermis and hyperkeratosis. ICD also shows a perivascular inflammatory infiltrate in the dermis. In epidermis, the inflammatory infiltrate characteristically adopts a nonfocal, diffuse distribution. There may be some spongiosis, but it is habitually associated with ballooning of epidermal cells (intracellular edema), a phenomenon characterized by abundant, pale-staining cytoplasm of keratinocytes. In addition, the intraepidermal vesicles often develop into vesiculo-pustules and there is dermal and epidermal infiltration of neutrophilic granulocytes (Avnstorp, 1988). Strong irritants may induce necrosis of keratinocytes. Chronic ICD is usually indistinguishable of chronic ACD, showing acanthosis and hyperkeratosis.

It has been suggested that spongiosis may be specific of allergic reactions. In a comparative light microscopic study, early allergic patch-test responses (6–8 h after challenge) were characterized by follicular spongiosis, although clinically equipotent irritant reactions induced by SLS showed no significant changes (Vestergaard et al., 1999). However, spongiosis has been observed after challenge with

TABLE 16.2
Histological and Histochemical Differences between ICD and ACD

	ICD	ACD
Histology	Epidermis: Moderate spongiosis, intracellular edema, exocytosis spread, diffuse distribution of the inflammatory infiltrate in epidermis	Spongiosis with microvesicles predominates
	Occasionally, neutrophil-rich infiltrates	Focal distribution of the inflammatory infiltrate the epidermis
	Pustulation and necrosis may develop. Greater pleomorphism	Pustulation is rare
CD1+Langerhans cells	Decreased	Initial decrease in number, then increase
Immunohistochemistry	CD4+ T cells predominate, some CD8+ T cells	CD4+ T cells predominate, some CD8+ T cells
	In activated state (IL-2 expression)	In activated state (IL-2 expression)
	Increased expression of ICAM-1 by keratinocytes (the results from different studies have been conflictive)	Increased expression of ICAM-1 by keratinocytes
	Increased expression of HLA-DR by keratinocytes (the results from different studies have been conflictive)	Increased expression of HLA-DR by keratinocytes
Epidermal volume (proliferation)	Increase in epidermal volume at 24 h after challenge. Proliferating epidermal cells reach a peak 4 days after challenge. Keratin 16 and involucrin expression in the epidermis increased rapidly after challenge reaching a peak after 3 days and fading thereafter	Increase in epidermal volume at 72 h after challenge. Keratin 16 and involucrin expression in the epidermis increasing more slowly reaching a peak 4 days after challenge

other irritants such as benzalkonium chloride, croton oil, and dithranol (Willis et al., 1989).

Avnstorp et al. (1988) selected 17 histological variables for establishing the differential diagnosis between irritant and allergic reactions. In ACD reactions, the focal disposition of the inflammatory infiltrate in epidermis was found to be significantly different from the widespread distribution in ICD reactions. Necrosis was a significant feature in the diagnosis of irritant reactions, as it was in the presence of neutrophilic granulocytes in the dermal stroma. Statistical analysis by correlation of the selected variables provided a diagnostic specificity of 87% and a sensitivity of 81% for allergic reactions. In irritant reactions the specificity was 100%, but the sensitivity was only 46%. By multiple regression analysis, an index could be calculated: $4 \times$ necrosis $- 3 \times$ edema $- 2$. Subzero values denoted irritancy, while values above zero indicated allergy.

Both allergic and irritant challenges induce epidermal proliferation; however, the dynamics are different. Irritant reactions to SLS induced a statistically significant increase in epidermal volume at 24 and 72 h after challenge, compared to 0 and 6 h ($p < 0.003$ and $p < 0.001$, respectively), whereas the increase in the epidermal volume in allergic reactions to nickel sulfate was not noted until 72 h after challenge, compared to 0, 6 ($p < 0.001$), and 24 h ($p < 0.004$) (Emilson et al., 1998). Le et al. (1995) observed that challenge with the irritant SLS induced a higher and earlier increase in the number of cycling epidermal cells, and the maximum proliferation was reached 4 days after challenge. In comparison, allergic reactions showed a gradual increase in proliferating cells until a peak was attained on day five. Similarly, the expression of keratin 16 (K16), a molecule that is present in the suprabasal epidermis under hyperproliferative conditions, and involucrin, a marker of terminal differentiation, increased rapidly following challenge with SLS, reaching a peak after 3 days and fading thereafter, while allergic reactions exhibited a more delayed response reaching a maximum after 4 days. A positive CD36 (OKM5) expression was found both in irritant and allergic patch tests (Vestergaard et al., 1995). It has been postulated that there may possibly be a connection between OKM5 expression in the stratum granulosum and the proliferative state of the epidermis (Willis et al., 1991).

Concerning the cells of the inflammatory infiltrate, identical composition of peripheral T lymphocytes, associated with peripheral HLA-DR positive macrophages and LC, is observed in ICD and ACD (Scheynius et al., 1984; Ferguson et al., 1985; Brasch et al., 1992; Ranki et al., 1983). In the lymphocyte population, helper/induced T lymphocytes exceed the number of suppressor/cytotoxic cells in both types of reaction (Scheynius et al., 1984; Avnstorp et al., 1987). The number of infiltrating cells was larger in biopsies from allergic reactions induced by nickel sulfate than in irritant reactions induced by SLS. However, the kinetics of the cell responses, the phenotypes of the inflammatory cells, their allocation, and spatial relationship was comparable (Scheynius, 1984). Similar findings were reported by Gawkrodger et al. (1986), although slight differences in the spatial distribution between both types of reactions were observed. Dermal infiltrates were larger in the allergic reactions, but epidermal invasiveness was greater in the irritant reactions. Using laser scanning microscopy and indirect immunofluorescence, Emilson et al. (1998) evaluated the epidermal expression of HLA-DR and the invariant chain reactivity associated with antigen processing and presentation in allergic and irritant reactions. No significant change in the epidermal volume of HLA-DR reactivity was found in both types of reactions, nor was any significant change in the epidermal volume of invariant change reactivity in the allergic reactions. In the irritant reactions, however, there was a significant decrease in the epidermal volume of invariant chain reactivity from 24 to 72 h. Also, 72 h irritant reactions had a significantly lower epidermal volume of invariant chain reactivity compared to allergic reactions. This decline might reflect an epitope-induced alteration by irritants or a downregulated biosynthesis of the invariant chain due to variance in local cytokine production between both types of inflammatory reactions.

Using confocal and electron microscopy, Rizova et al. (1999) showed that freshly isolated human LC preincubated with contact sensitizers internalized the HLA-DR molecules, preferentially in lysosomes situated near the nucleus, whereas the irritant-treated or -untreated LC internalized these molecules in small prelysosomes located near the cell membrane.

16.4 ALLERGENS AND IRRITANTS IN IMMUNOTOXICOLOGY TESTING

During the past two decades growing interest arose in developing improved toxicological methods for differentially diagnosing contact allergic reactions from contact irritant reactions in humans. The majority of tests for predicting allergenicity of chemicals use guinea pigs or mice with biphasic protocols, comprising a sensitization phase (induction) and an elicitation phase (challenge) (Buehler, 1965; Magnusson and Kligman, 1969; Maurer et al., 1980). In guinea pig models, such as the guinea pig maximization test, Buehler's occluded patch test, or the optimization test, contact reactivity is assessed mainly by a subjective local erythema score and determined by the frequency of animals exhibiting a positive response. In the challenge-induced mouse ear–swelling test (MEST), induration is the predominant feature of the positive allergic reaction. To avoid false–positive results, challenge must be performed with a concentration of the test material that is unable to provoke skin irritation in nonsensitized controls. It is possible, therefore, with highly irritant materials that the concentrations selected for challenge are below those necessary to elicit a contact hypersensitivity reaction. More recently, the murine local lymph node assay (LLNA) was described as an alternative method for the detection of moderate or strong contact sensitizers. In contrast to guinea pig models and the MEST, the LLNA is based upon the detection of a primary immune response as a function of the cell proliferation in the draining lymph nodes after epicutaneous exposure to materials (Kimber et al., 1986; Kimber and Weisenberger, 1989; Basketter et al., 1991, 1994).

Although the LLNA was first described to selectively detect allergic skin immune responses, recent studies showed that many irritant compounds like sodium dodecyl sulfate (SDS), chloroform/methanol, triton X-100, croton oil, benzalkonium chloride, salicylic acid, dimethyl sulfoxide, among others, also induce node cell proliferation not distinguishable from the results with low-grade or moderate irritants (Montelius et al., 1994; Ikarashi et al., 1993; Homey et al., 1998). Therefore, irritant substances could be wrongly classified as allergens, or the allergenicity of substances with both allergenic and irritant properties could be overestimated.

To differentiate the cellular and molecular events elicited by allergens and irritants, Sikorski et al. (1996) performed a phenotypic analysis of lymphocyte subsets in the draining lymph nodes after topical treatment. The expression of CD3, CD4, CD8, and B220 was evaluated by flow cytometry. The allergens oxazolone and trinitro-chloro-benzene (TNCB) and the irritant benzalkonium chloride increased the total number of T and B lymphocytes in comparison with vehicle. In allergen-treated mice, a preferential increase in B lymphocytes, as seen by an increase in B220+ cells, was apparent in comparison with those treated with irritant and vehicle. This increase was consistently more prominent when stronger sensitizers or higher concentrations were used. Specific markers of antigen-induced T-cell activation were also examined (Gerberick et al., 1997). CD62L (L-selectin) and CD44 (H-CAM) expression was selectively modulated after treatment with the contact allergen TNCB when compared with the irritant benzalkonium chloride or the vehicle control. Mice treated with dinitro-chlorobenzene (DNCB) also had an increase in the percentage of CD4+ cells expressing $CD62L^{lo}CD44^{hi}$ that were dose dependent and peaked at 72 h after the final allergen treatment. In addition, increases in the percentage of CD8+ cells expressing $CD62L^{lo}CD44^{hi}$ were observed with allergens, including DNCB, oxazolone, and hexylcinnamic aldehyde, but not with irritants (Gerberick et al., 1997). Homey et al. (1998) observed that topical treatment with allergens (1% oxazolone) strongly induced the activation markers CD25 and CD69 on CD4+ and CD8+ lymph node cells, whereas irritants (croton oil) induced only marginal upregulation. Furthermore, treatment with oxazolone resulted in an expansion of I-A+/B220+ and a significant upregulation of CD69 on I-A+ lymph node cells. In contrast, croton oil treatment resulted in only a minor increase of both I-A+/B220+ and CD69/I-A+ lymph node cell subpopulations. Differences were considered to be qualitative rather than quantitative, since the analyses were performed with oxazolone and croton oil concentrations that induced comparable lymph node cells count indices. The upregulation of CD69 on I-A+ lymph node cells, confirming antigen-specific sensitization, was the most prominent marker for differentiation between the reaction patterns of oxazolone and croton oil. Phenotypic analyses of the CD69/I-A+ lymph node cells revealed them to be predominantly B220+ cells. Thus, examination of cellular phenotypic changes by flow cytometry demonstrates to be valuable in differentiating between allergic and irritant responses in the draining lymph nodes of mice.

Further criteria for the differentiation between allergens and irritants using a modified LLNA were proposed by Homey et al. (1998). They postulated that contact allergens induce activation and proliferation of skin-draining lymph node cells with only marginal skin inflammation, whereas irritants predominantly induce skin inflammation and, as a result, generate lymph node cell proliferation. Therefore, a differentiation index (DI) was developed considering the relationship between local draining lymph node activation (calculated as a percentage of the maximal increase in lymph node cell count index) and skin inflammation (calculated as a percentage of maximal ear swelling). DI values >1 indicated an allergic reaction pattern, whereas DI values <1 denoted an irritant response. The sensitizing potential of oxazolone was clearly confirmed eliciting a DI ranging from 2.5 to 16.6 in three different experiments. In contrast, the irritant croton oil produced a DI varying from 0.7 to 0.8 (Homey et al., 1998).

An increased understanding of the immunologic events that mediate and regulate allergic and irritant skin responses may provide a mechanistic basis for development of more objective and accurate *in vivo* and *in vitro* models of immunotoxicology testing.

16.5 CLINICAL DIAGNOSIS

Irritant dermatitis is not a single clinical entity but rather a heterogeneous spectrum of disorders (Table 16.1) with diverse pathophysiology and a broad range of clinical and histological features (Berardesca, 1997). The diagnosis of acute ICD to strong skin irritants agents is usually straightforward, because of the rapid onset of skin lesions after exposure points to the causative agent. However, subacute or chronic contact dermatitis frequently appears as an eczematous condition without a readily apparent cause. In these circumstances, the clinician must go through a decision process to discriminate among ICD, ACD, and other eczematous conditions. Differentiation between chronic ICD and ACD is frequently impossible on the basis of macroscopic or microscopic morphology (Table 16.2). The clinical picture in both conditions may include erythema, edema, lichenification, vesicles, bullae, oozing excoriations, scaling, and hyperkeratosis. Pustules, necrosis, and ulceration may be seen after contact with strong irritants, whereas they are rarely observed in ACD. Vesiculation, edema, and oozing are usually more prominent in ACD. Symptoms of ICD may include burning, pain, itching, stinging, soreness, and discomfort. Pruritus is the cardinal symptom in ACD. ICD lesions are usually sharply demarcated and confined to the contact area; while in ACD, lesions are poorly circumscribed and frequently disseminated. However, ICD lesions may disseminate depending on the characteristics of the exposure. Irritant reactions were believed to be unspecific and reproducible in all exposed subjects, in contradistinction with the uniqueness and specificity of allergic reactions. Yet, different irritants produce inflammation by different mechanisms and through d ifferent mediators. The effects of irritants on cutaneous targets depend on several factors such as the type of chemical, concentration, mode of exposure, concomitant environmental

factors, and individual susceptibility (Lammintausta et al., 1987; Berardesca and Maibach, 1988; Lammintausta and Maibach, 1988; Wilhelm and Maibach, 1990; Patil and Maibach, 1994; Judge et al., 1996; Rietschel, 1997). Irritant thresholds and dose responses vary considerably among individuals when tested with a low concentrated or mild irritant and also in the same individual over time (Judge et al., 1996).

Concerning the timing of the dermatitis, features that claimed helpful in distinguishing irritant dermatitis include cutaneous reaction upon first exposure—at least with potent irritants—and rapid onset of dermatitis after exposure. In ACD, two phases are required: an induction phase, during which sensitization is acquired; followed by elicitation of a cutaneous inflammatory reaction in subsequent exposures. Usually, the induction phase does not result in clinical skin lesions, probably due to the low numbers of responder T lymphocytes present. Subsequent challenges, resulting in clonal T-cell expansion and representation of the antigen to already primed (memory) T cells, may induce an enhanced inflammatory response eliciting the clinical dermatitis. However, a potent allergen may induce both sensitization and elicitation phases through a single exposure. Acute ICD develops rapidly after exposure, while ACD lesions usually appear 24–96 h after the last exposure to the causative agent, depending on the characteristics of the sensitizer, the conditions of exposure, and the individual susceptibility.

Patch testing is applied to make the distinction between ACD and ICD in clinical settings. The morphology and kinetics of the response is important when assessing patch-test reactions. The irritant reaction usually reaches its peak quickly after exposure, and then starts to heal; this is called the *decrescendo* phenomenon. Allergic reactions are characteristically delayed, reaching their maximum at approximately 72–96 h (*crescendo* phenomenon). However, some irritants may elicit a delayed inflammatory response, and visible inflammation is not seen until 24 h or even more after exposure (Malten et al., 1979; Bruynzeel et al., 1982; Lammintausta and Maibach, 1990; Reiche et al., 1998).

16.6 CONCLUSIONS

The current understanding of mechanisms of both irritant and allergic dermatitis does not allow for establishing pertinent and practical criteria for a clear-cut differentiation between them. Although the pathways for ICD and ACD are distinctly defined, there seems to exist an overlapping and interconnected cellular and molecular network between both types of contact dermatitis. Therefore, differences between irritants and allergens are more conceptual than verifiable.

Further understanding of the molecular pathways in contact dermatitis would be significant in dermatological practice, as well as in clinical and toxicological research.

A better comprehension of the immunologic events that mediate and regulate allergic and irritant skin responses may provide opportunities for development of more objective and accurate methods in clinical practice and toxicological research.

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17 Occupational Allergic Contact Dermatitis: Rational Work-Up

Iris S. Ale and Howard I. Maibach

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17.1 INTRODUCTION

Occupational contact dermatitis (OCD) is an inflammatory skin disorder resulting from cutaneous contact with materials primarily associated with the workplace. OCD is an important medical and occupational health problem, with incidence rates ranging from 24/100.000 to 170/100.000 (Cherry et al., 2000; Diepgen and Coenraads, 1999; Mathias and Morrison, 1988). Of work-related cases involving the skin, 90–95% will involve contact dermatitis (Lushniak, 2003; Mathias et al., 1990). Actually, most cases of contact dermatitis treated by physicians are in some way work related. OCD can be broadly classified into irritant and allergic. However, OCD is often multifactorial, and the different contributory factors can be difficult to ascertain. The reported frequency of occupational allergic contact dermatitis (OACD) and occupational irritant contact dermatitis (OICD) in different studies varies, depending on differences in how occupational diseases are notified and recognized, the specific characteristics of the occupation, the comprehensiveness of the workplace investigation, and the medical examination, including the accuracy of diagnostic patch testing.

Medical management has had little impact on clinical outcome in OCD, and most studies report persistent disease in 33–81% of the individuals (Emmett, 2003; Kanerva et al., 1995). Establishing suitable therapeutic and preventive measures requires an accurate diagnosis and the identification of all causal or contributing agents. A more comprehensive approach to prevention and management, based on rational criteria, is required to substantially reduce the burden of OCD.

17.2 DIAGNOSIS OF OACD

The diagnosis of OACD depends on patient history, dermatitis pattern, occupational exposure history—including hazard identification, estimation of dermal exposure, and risk characterization—and comprehensive diagnostic patch testing. It involves two crucial elements: (1) recognizing the existence of an occupational exposure and (2) assessing whether that exposure can be linked to the expression of the dermatitis process as a cause or substantial contributive factor. Even though exposure to an occupational allergen is the essential factor, whether or not this exposure will finally result in clinical dermatitis, depends on additional circumstances, therefore, the rational work-up should include the analysis of all predisposing and contributory factors.

17.2.1 Clinical History

The first step in establishing a work exposure as the cause of contact dermatitis is to take a detailed clinical and occupational history (Table 17.1). A dermatitis that clears or improves substantially during a 2–3 week break from work and recurs within a few days of the return to work strongly suggests an occupational origin. OACD tends to recede over a period of 3 or more weeks after the contact allergen is withdrawn. Significant improvement in the course of a few days away from work may occur with weak irritant reactions, but it is improbable with allergic reactions. Often OACD improves more slowly than OICD when the occupational exposure ceases and recurs faster (in a few days) when exposure is restored. Contrarily, irritant dermatitis to several weak

TABLE 17.1
Clinical History for the Assessment of OACD

Clinical characteristics of the dermatitis
- Time of onset and possible relationship with the occupation
- Characteristics of initial lesions and clinical evolution
- Dermatitis area corresponding to exposure site and occupational gestures
- Dermatitis morphology suggesting specific allergens

Time relationship to occupation. Effect of holidays and time-off work

History of occupational exposure
- Job description. Occupational gestures and characteristics of the working milieu
- Potential allergens and irritants in the working environment
- Characteristics of the exposure: dose, frequency, site
- Concomitant exposure factors: temperature, humidity, occlusion, friction, etc.
- Personal protective measures at work (gloves, masks, barrier creams)

Other workers similarly affected

History of nonoccupational exposure
- Domestic products: cleanser, detergents
- Skin care products, fragrances, nail and hair products
- Pharmaceutical products (prescription and over the counter)
- Personal protective measures at home (gloves)
- Jewelry, clothing
- Homework and hobbies

Special variants on exposure
- Photoexposure
- Indirect contact (fomites, connubial exposure)
- Airborne contact

History of previous dermatitis, atopy, and other skin/general diseases
- Past contact dermatitis (occupational or not)
- Previous patch testing
- Other exogenous or endogenous dermatitis: atopic dermatitis, stasis dermatitis, psoriasis, sensitive skin
- Atopy (asthma, rhinoconjunctivitis)

Family atopy and other skin diseases

irritants, that is, cumulative irritant contact dermatitis, usually requires many weeks to reappear when exposure is reestablished. A time course of 2–4 days between exposure and recurrence is suggestive of contact allergy. However, the elicitation time depends on the characteristics of the sensitizer, intensity of exposure, and degree of sensitivity. Contact allergic reactions will not appear until at least 2 weeks after the sensitizing exposure in a new worker—or following the introduction of a new allergen in the working environment—since this is the minimum sensitization period for most allergens. In contrast, reactions to strong irritants do not require an induction period and may appear within minutes to hours after the first exposure. Usually, several exposures are required for an OACD to develop. Weak irritants may mimic the effect of allergens, inducing dermatitis after a series of exposures.

Clinical history should investigate all possible sources of allergenic exposure in the workplace and should also scrutinize nonoccupational sources, comprising hobbies and domestic exposures: for example, personal skin care products and fragrances, medicaments, clothing, and accessories. Sometimes, even when a comprehensive history has been taken, the source of allergenic exposure remains concealed. Many times patients fail to recognize a causal allergen when the exposure is infrequent or sporadic. In addition, exposure may be indirect, that is, the contact may be airborne, ectopic, connubial, or through a contaminated item. Furthermore, some sources of allergenic exposure remain hidden due to insufficient chemical identification in many household or industrial products.

Finally, clinical history should provide insights in differentiating OACD from other exogenous or endogenous dermatoses, such as atopic dermatitis or psoriasis.

17.2.2 Clinical Examination

OACD is usually characterized by eczematous inflammation. However, it is frequently difficult to distinguish OACD from other forms of dermatitis, especially irritant contact dermatitis on the basis of exclusive morphological criteria. No pathognomonic clinical signs and symptoms can unambiguously discriminate between allergic and irritant contact dermatitis, although vesiculation and pruritus are more frequent in the former. To further complicate the matter, most allergens also have irritant properties, and many times dermatitis is the result of both irritancy and allergy. Sometimes, clinical examination may reveal an occupational "mark" that points out to a specific occupational exposure. Most of the time, the physician must rely on the pattern of distribution and general characteristics of the dermatitis to determine the causative agent. For instance, eczematous lesions affecting the exposed areas of the face, eyelids, and skin folds are commonly observed in airborne dermatitis from fumes, dusts, or other airborne particles. Compromise of the dorsal aspects of the hand and fingers, and especially, the finger webs is frequently observed in wet work occupations. However, the original pattern of the dermatitis is frequently modified by secondary dissemination, secondary infection, treatment etc., requiring a sensitive diagnostic approach.

17.2.3 Patch Testing

The strongest evidence that an allergic contact dermatitis is of occupational origin is a positive patch test to a nonirritating concentration of a chemical found in the workplace, which plausibly could come into contact with the areas of dermatitis. Habitually, the patch-test evaluation of subjects with suspected OACD starts with one of the standard screening series of allergens, such as the one proposed by the International Contact Dermatitis Research Group (ICDRG) (Lachapelle et al., 1997), supplemented by additional series tailored for specific occupations, as well as any other allergen that may be associated with the clinical situation. Testing with additional allergens depending on the exposure and specific occupations gives a substantial number of additional positive reactions (Menné et al., 1992). Also, testing with substances used by the patient in the workplace is often required. Many of these nonstandard occupational materials have irritant properties, and special caution should be exerted when testing with them. Besides producing false-positive irritant reactions, the

injudicious use of undiluted industrial substances in patch testing increases the risk of active sensitization.

As a rule, patch testing should be performed with the highest nonirritating concentration of the chemical. When possible, information about appropriate concentrations and vehicles for nonstandardized substances should be obtained. If no information can be found, an open (unoccluded) test should be done using a low concentration of 0.1–2.0% of the putative substance(s) in a nonirritant vehicle (water, petrolatum, etc.). If no irritation appears with increasing dosage, an occluded test may be performed. It is advisable to incorporate a dose–response assessment performing the patch test with serial dilutions of the substance.

False-negative reactions are especially treacherous since the cause of the dermatitis may be missed. Many times, false-negative reactions are due to methodological or technical problems; for example, insufficient occlusion and other technical flaws, concurrent treatment with corticosteroids, and failure to perform delayed readings. However, in occupational dermatology, the most significant cause of false-negative reactions is the failure of the patch test to reproduce the actual conditions of the occupational exposure: (1) sweating and mechanical trauma experienced at work may not be adequately reproduced in patch testing; (2) the allergen concentration in the products obtained from the workplace may be insufficient to elicit an allergic patch-test reaction following a single application, but enough to produce an OACD after multiple occupational exposures; (3) insufficient cutaneous penetration of the allergen: allergens are applied on normal skin in patch testing, but, in the real life situation, a pre existent work-related irritant dermatitis may predispose to the development of contact allergy. When patch-testing results are negative, but the suspicion of OACD persists, it is helpful to carry out additional tests, such as a repeated open application test (ROAT), a provocative use test (PUT) (Nakada et al., 2000; Villarama and Maibach, 2004), or testing with product's extracts. Occasionally, photopatch testing should be performed to rule out the possibility of photosensitization to an occupational substance. Contact urticaria to work-related materials is also possible, since immediate contact reactions may lead to dermatitis. This association is especially important in workers exposed to latex gloves and also in the food industry. Immediate hypersensitivity testing is mandatory when contact urticaria or protein contact dermatitis is suspected.

17.2.4 Assessment of Clinical Relevance

The fact that contact allergy to one or several allergens has been reliably demonstrated by careful patch testing does not guarantee that these allergens are responsible for the patient's dermatitis. It must be established whether or not the responsible allergen is relevant to the dermatitis, either as a primary cause or as an aggravating factor. Our goal in assessing relevance is to ascertain the putative responsibility of a particular allergen to the clinical circumstance. In this sense, the exposure to the incriminated allergen may explain the dermatitis entirely, that is, "complete relevance," but dermatitis with a multifactorial background frequently occurs.

Assessing the relevance of a positive patch-test reaction is often complicated and involves many confounding factors. "Evaluating the relevance of a reaction is the most difficult and intricate part of the patch test procedure, and is a challenge to both dermatologist and patient. The dermatologist's skill, experience and curiosity are crucial factors" (Wahlberg, 1995). If an allergen produces a patch-test reaction and the patient is exposed to circumstances in which skin contact with occupational materials known to contain the allergen is likely to occur, relevance is considered possible. If a PUT or a ROAT with the product to which the patient is in contact—and it is known to contain a sufficient amount of the allergen to elicit the dermatitis—produces a positive response, relevance can be considered certain. Absolute proof of relevance is often unattainable, and many times a positive reaction is judged nonrelevant because of insufficient environmental information. Frequently, it is difficult to substantiate the presence of the allergen in the patient's environment. This may be due to the complexity in detecting certain allergens or to insufficient knowledge about the composition of different products. As a consequence, the relevance scores for different allergens vary; the easy the identification of the source of an allergen, the higher the relevance scores (Ale and Maibach, 1995).

Guidelines for assessment of relevance have been proposed (Table 17.2). Assessment starts with a comprehensive clinical history and physical examination and should be supplemented by a rigorous environmental evaluation,

TABLE 17.2
Suggested Guidelines for the Assessment of Relevance

1. Revise the clinical history
   - Reinterrogate the patient in light of the test results
2. Look for all possible sources of allergen exposure
   - Perform a workplace visit
   - Consider all possible types of exposure: direct, indirect, sporadic, concealed, etc. Consider cross-reacting substances
   - Obtain information from "lists" of allergens, databases, products' manufacturers, etc.
   - Perform chemical analysis of suspected products
3. Assess the sensitization potential of the substance
   - Obtain data from predictive tests, structure/activity analysis, and epidemiological studies
4. Assess all exposure parameters
   - Concentration of the substance in the suspected product
   - Route of exposure
   - Specific site of contact and possible presence of skin damage or dermatitis in the contact area
   - Intensity of exposure (i.e., dose, duration, frequency, and total surface area)
   - Simultaneous exposure factors: humidity, occlusion, temperature, mechanical trauma
5. Perform additional testing procedures with the suspected allergen(s), products brought by the patient presumably containing the suspected allergen or product's extracts

TABLE 17.3
Testing Procedures for the Assessment of Relevance

1. Testing with the suspected allergen(s)
   - Sequential patch testing
   - Repeated open application test (ROAT) or provocative use test (PUT)
     - on normal skin
     - on slightly damaged or previously dermatitic skin
2. Testing with products suspected to contain the responsible allergen
   - Patch testing (using suitable vehicle and appropriate concentration, frequently starting with highly diluted substances)
   - ROAT (similar as stated earlier, using proper vehicle and adequate concentration)
   - PUT (typical product use)
   - Testing in normal controls (if necessary)
3. Testing with product's extracts
   - Similar to 2
4. Testing with cross-reacting allergens and products suspected to contain them
   - Similar to 1

TABLE 17.4
Exposure Assessment in OACD

Characteristics of the suspected substance or product
- Assessment of the hazardous potential of the substance or product
  - Data procurement from predictive assays for delayed hypersensitivity (e.g., quantitative structure–activity relationship, murine local lymph-node assay, guinea pig sensitization test, and human skin sensitization assays)
  - Data from epidemiological studies
- Assessment of the physicochemical properties of the substance (i.e., pH, solvent properties, hygroscopicity, oxidizing capacity, substantivity, binding capacity, and wash-and-rub resistence to removal)
- Concentration of the substance

Characteristics of the exposure
- Routes of exposure
  - Skin exposure: specific cutaneous sites, previous skin damage
  - Other routes: respiratory, gastrointestinal
- Intensity of exposure
  - Dose
  - Duration
  - Frequency (periodicity)
  - Specific site of contact and area of exposure
- Concomitant exposure factors (i.e., wet work, occlusion, temperature, humidity, and mechanical trauma)

investigating the existence of exposure to the putative agent, characteristics of this exposure, and possible concurrent factors. Visiting the patient workplace enables the physician to obtain a comprehensive picture of the real conditions at the working environment, bringing many details into clinical significance. Additional tests such as testing with product's extracts, ROAT, PUT, etc. may prove valuable in establishing a definite causative relationship (Ale and Maibach, 1995; Marrakchi and Maibach, 1994) (Table 17.3). The positive patch-test reactions for which clinical relevance cannot be established may represent false-positive results. However, much too frequently they represent true-positive reactions, wherein the patient fails to recall a significant exposure or the clinician omits to retrieve the pertinent historical data, to trace the responsible environmental exposure, or to perform the appropriate tests. Relevance has been defined as the capability of an information retrieval system to select and redeem data appropriate to a patient's need (Lachapelle, 1997).

17.2.5 Assessment of Exposure

Exposure assessment is an important analytical tool in occupational dermatology for evaluating the likelihood and extent of actual or potential exposure of workers to the source of a chemical hazard. The field of exposure assessment has developed significantly in the past 15 years, and exposure assessment techniques are now available, which can significantly improve the quality of epidemiologic and health risk assessment studies (Jayjock, 1998). Better quantitative exposure components can now be incorporated into these studies, such as measuring the concentration of many chemicals to which workers are exposed, cataloguing the various exposure pathways and routes, and identifying typical values, which can be used in the exposure calculations. In addition, a number of exposure assessment models are available for estimating personal exposure to certain chemicals of concern in both the workplace and the general population. The goal of exposure science is to identify and characterize "real world" contacts with exogenous materials and uptake in the body of toxic materials that can cause acute or chronic health effects. Exposure science is predominantly observational, performed in the field within normal living and working situations. The knowledge obtained can then be used in computer models for generalization to other populations including people deemed to be at higher risk. The conclusions drawn from these studies allow the evaluation of public health and environmental policy options for effective reduction or prevention of exposure.

Industrial exposures generally occur via the dermal or respiratory routes. Gastrointestinal exposure may result from contaminated hands or handling of foods, but it is uncommon in occupational settings. Direct skin contact with the causative agent represents the most relevant route of exposure for occupational dermatitis. Additionally, dermatitis may result following inhalation in a sensitized worker. Likewise, induction of skin sensitization may result in subsequent heightened respiratory responsiveness following inhalation exposure (Arts et al., 2006).

It is essential to be informed about the hazardous properties of all the suspected occupational substances, including their intrinsic sensitizing capacity, as well as their physicochemical properties (Table 17.4). The sensitizing potential of occupational or consumer products regularly follows a stepwise approach that may involve structure–activity evaluations (Hostynek et al., 1996), analytical assessments, preclinical skin sensitization testing (e.g., the murine local lymph node assay (Basketter and Cadby, 2004; Dearman et al., 1999;

Kimber et al., 2001), guinea pig sensitization tests (Maurer et al., 1994), confirmatory clinical testing (e.g., the human repeat insult patch test [HRIPT], human maximization tests) (Schneider and Akkan, 2004), and benchmarking of resulting data against similar substances and product types (Gerberick and Robinson, 2000). In addition to the intrinsic properties of the substance, quantitative data regarding exposure and other concomitant factors should be evaluated, including: (1) specific cutaneous site and total area of exposure (Upadhye and Maibach, 1992), (2) existence of skin damage or dermatitis in the contact area, (3) duration and periodicity of the exposure, (4) concentration and dose of the causative agent, and (5) additional exposure factors (Table 17.2). Cutaneous site and total area of exposure are significant factors in determining the skin penetration of the substances (Wester and Maibach, 1985). Skin damage or irritation may result in a higher cutaneous permeability and a significant reduction of the no-effect level for a specific compound. Therefore, the clinical scenario will often show that an OACD is preceded by an OICD. The intensity of the exposure, that is, dose, duration, frequency, and total area of exposure is a significant factor. Sensitization and subsequent dermatitis may result from a single exposure to a very strong allergen. However, repeated exposures are usually required before sensitization occurs. Contact allergy is a dose-related phenomenon, and there is a threshold surface concentration of the allergen required to induce sensitization or elicitation of the response (Basketter et al., 1997; Boukhman and Maibach, 2001; Kimber et al., 1999; Marzulli and Maibach, 1976). Reported results suggest that especially relatively high concentrations can induce sensitization, and that prevention of such concentrations will prevent workers from developing occupational allergies. The threshold concentration for the elicitation of allergic skin reactions in sensitized subjects is generally lower than the threshold to induce sensitization (Scott et al., 2002). Therefore, it is important to consider the low threshold levels for elicitation for recommendation of health-based occupational exposure limits. Despite the observation of dose–response relationships and no-effect levels, due to a number of uncertainties, no definite conclusions can be drawn about absolute threshold values for allergens with respect to sensitization and elicitation reactions in the skin, as well as the respiratory tract. In predictive tests, it was observed that the threshold of elicitation decreases as the doses used to induce the sensitization increase, making difficult to establish clear threshold doses below which allergic responses are not seen (Hostynek and Maibach, 2004). Besides, most predictive tests are generally intended to detect the potential of a chemical to induce skin or respiratory allergy at relatively high doses. As a result, these tests do not provide information of dose–response relationships at lower doses such as those observed in occupational situations. In addition, dose–response relationships and threshold values for occupational allergens have been obtained by a broad variety of test methods using different techniques, for example, intradermal exposure, topical exposure, and inhalation exposure at the workplace or different endpoints (Arts et al., 2006; Robinson et al., 2000). Therefore, standardized and validated dose–response test methods are urgently required to be able to recommend safe exposure levels for allergens at the workplace.

17.3 CONCLUSION

As stated earlier, assessing OACD frequently represents a complex and multifaceted procedure. A rational work-up includes eight consecutive steps as follows:

1. Identification of occupational exposure to sensitizing substance(s).
2. Demonstration of time relationship between the occupational exposure and the clinical course of dermatitis.
3. Physical characteristics of the dermatitis consistent with the occupational exposure regarding the morphology and localization.
4. Nonoccupational exposure excluded (not present or irrelevant).
5. Positive patch test with the sensitizer in appropriate vehicle and concentration.
6. Positive PUT or ROAT with the sensitizing materials: allergens, allergen-containing products, or product's extracts.
7. Identification of the sensitizer in the suspected occupational products in sufficient concentration to elicit the dermatitis.
8. Clearing of the dermatitis when the allergen is removed from the occupational environment or the exposure is substantially decreased.

Nonsingle criterion provides sufficient evidence for the probable occupational origin of allergic contact dermatitis. OCD is often of multifactorial origin, and it is difficult to determine the relative significance of the various contributing factors. Diagnosis of OACD requires a high index of suspicion and knowledge of the worker's environment, as well as a judicious clinical evaluation including comprehensive skin testing. The correct diagnosis and identification of the occupation-related allergen improves the primary, secondary, and tertiary preventive strategies for OACD. If undiagnosed, the disease may persist and deteriorate, resulting in permanent disability to the worker.

With a better knowledge of the chemical environment and more advanced test techniques, it is probable that more cases of contact dermatitis will eventually demonstrate to have an occupational allergic component. Also, epidemiologic animal and human studies are necessary to determine the capacity of the chemicals used in industry to induce OACD. It is necessary to standardize the predictive tests to compare results among laboratories and to predict the potential for sensitization. Exposure–effects relationships should be established with increased certainty to recommend safe exposure levels for allergens at the workplace. The above guidelines can provide a simplified rational approach for assessing OACD until more information is forthcoming.

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18 Systemic Toxicity

Philip Hewitt and Howard I. Maibach

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18.1 INTRODUCTION

Human skin is exposed to a number of chemicals and drugs throughout our entire lives. Following percutaneous absorption, a chemical and or its metabolites may cause toxicity locally or in another organ, distant from the point of entry. Although not generally appreciated, some chemicals are more toxic, at least in animals, when applied topically rather than orally. Furthermore, many compounds are absorbed to a greater degree from the skin than gastrointestinal (GI) tract, and whole body exposure can produce systemic absorption up to grams of material. This chapter focuses on the limited epidemiologic material available and depends mostly on case reports. Many compounds dermally absorbed are capable of producing systemic side effects whose occurrence and severity depends largely on the many factors that can affect the absorption of topically applied compounds (both physiological/pathological condition of the skin and physicochemical properties of the compound). The majority of reports for systemic toxicity have been from industrial chemicals/agrochemicals and occupationally these probably have the greatest potential hazard after dermal exposure.

18.2 FACTORS AFFECTING PERCUTANEOUS ABSORPTION

18.2.1 Integrity of the Barrier

The stratum corneum layer of the epidermis is a major barrier to percutaneous absorption. Anything that alters the structure or function of the stratum corneum will affect epidermal absorption. The integrity of this barrier is reduced by any inflammatory process of the skin, such as any form of dermatitis or psoriasis, which may result in increased percutaneous absorption. Similarly, removal of the stratum corneum by stripping or damage by alkalis, acids, etc. will increase absorption.

18.2.2 Physicochemical Properties of the Substance

Percutaenous absorption is affected by the relative water/lipid solubility of the drug and the comparable solubility of the drug in its vehicle and in the stratum corneum. For a chemical to penetrate through the skin into the systemic circulation, it requires both a degree of lipophilicity (to facilitate its entry into the stratum corneum) and hydrophilicity (to aid its passage through the viable epidermis and dermis). Other factors such as molecular weight, molecular volume, and melting point will also be important determinants.

18.2.3 Occlusion

The penetration of some compounds can be increased by the use of an occlusive covering, up to 10-fold or more. This can be due to increased water retention in the stratum corneum, increased blood flow, increased temperature, and increased surface area after prolonged occlusion (skin wrinkling). Occlusion also prevents the accidental wiping off or evaporation (for volatile compounds), hence maintaining a higher local concentration on the skin surface.

18.2.4 Vehicle

The greater the affinity of a vehicle for the drug it contains, the lesser the expected percutaneous absorption will be. The physical properties of vehicles, especially the degree of occlusion they produce, affect percutaneous absorption, as discussed above (e.g., grease). Structural or chemical damage to the barrier layer can also affect the absorption rate; vehicles such as dimethyl sulfoxide cause greatly increased percutaneous absorption due to stratum corneum damage. In general, a higher concentration of the drug in its vehicle enhances penetration. Enhanced solubility produces greater thermodynamic activity yielding greater flux.

Extensive evidence on factors affecting penetration has been published.[1–3]

18.2.5 Anatomical Site

Regional differences in permeability of skin largely depend on the thickness of the intact stratum corneum.[4] According to the findings of a study by Feldmann and Maibach,[5] the highest total absorption of hydrocortisone is that from the scrotum, followed (in decreasing order) by absorption from the forehead, scalp, back, forearms, palms, and plantar surfaces. This large variation has also been shown for certain pesticides.[4] Variations in absorption with anatomical site have also been reported for other species, other than man, for example, monkey skin[6] and rat skin.[7]

18.2.6 Age

The greatest toxicological response to topical administration has been seen in the young. The preterm infant does not have an intact barrier function and hence is more susceptible to systemic toxicity from topically applied drugs.[8,9] A normal full-term infant probably has a fully developed stratum corneum with complete barrier function.[10] Yet, topical application of the same amount of a compound to both adult and newborn results in greater systemic availability in the newborn.[11] This is because the ratio of surface area to body weight in the newborn is three times that in the adult. Therefore, given an equal area of application of a drug on to skin of the newborn and adults, the proportion absorbed per kilogram of body weight is much more in the infant. Barrett and Rutter[12] and Maibach and Boisits[13] provide extensive documentation on this issue. Although counterintuitive, absorption of some compounds decreases in the aged.[14] Later Roskos and Maibach[15] reported that absorption was decreased in older subjects for steroids, but unchanged for other more hydrophilic compounds. They suggested that this was due to the decreased concentration of surface lipids in older subjects. Additionally, the dermal–epidermal junction changes with age, so that the blood circulation of the skin decreases.

18.2.7 Species Variation

Mammalian skin from different species is well known to exhibit great variation in percutaneous absorption. Factors such as stratum corneum thickness, hair follicle and sweat gland number, and the condition of the skin will play role. The distribution of blood supply and sweating ability differs between lab animals and man, therefore affecting absorption through the skin.[16,17]

18.2.8 Temperature

Generally, increased skin temperature enhances penetration rate.[18] This may be due to the increase blood flow associated with increased skin temperature or an increase in skin hydration.[19,20]

18.2.9 Metabolism

It has been well documented that the skin is capable of metabolizing a wide range of xenobiotics and has a full complement of phase I and phase II enzymes. The specific activities found in the skin are relatively low when compared to their equivalent hepatic forms. However, when the total volume of the skin is taken into account, then it is apparent that the skin is an efficient drug-metabolizing organ. This may have implications for the risk assessment of topically applied compounds, as metabolism will determine what form of the compound the systemic circulation will be exposed to. Cutaneous metabolism may also aid/impede percutaneous absorption of certain compounds. Detailed information on skin metabolism can be found in the review by Hotchkiss.[21]

18.2.10 Miscellaneous

Skin hydration, application time, concentration of the compound, particle size, skin injuries/condition, race, sex, and circulatory conditions have all been reported to affect the percutaneous absorption of dermally exposed compounds.

18.3 SYSTEMIC SIDE EFFECTS CAUSED BY TOPICALLY APPLIED COMPOUNDS

Topically applied drugs, cosmetics, and chemicals can cause allergic or irritant contact dermatitis. However, this type of side effect, usually limited to the skin, is outside the scope of this chapter. The reader is referred to the textbooks of Fisher[22] and Rycroft[23] for references to contact dermatitis as well as other chapter within this book. Systemic side effects from topically applied chemicals can sometimes result from either a toxic (irritant) reaction or a hypersensitivity reaction. The latter can be an anaphylactic type of reaction which is the extreme manifestation of the contact urticaria syndrome.[24] Many topical drugs and cosmetics have reportedly caused anaphylactic reactions. While anaphylactic reactions to topical medicaments are uncommon, their potentially serious nature warrants attention. However, reports of toxic (as distinct from allergic) reactions to applied drugs, cosmetics, or chemicals are more numerous and include many medicaments that have been safely used for many years, but which can be toxic under special circumstances.

The following is a short summary of the chemicals that have been reported to cause systemic side effects after topical application or accidental exposure.

18.3.1 Agrochemicals

It has been proposed that the most serious occupational skin exposure hazard is in agricultural workers involved in pesticide application. Contaminated clothing, lack of adequate protection, and unsafe spraying procedures have caused numerous toxic responses, mainly due to skin absorption.[25] Systemic toxicity after topical exposure to agrochemicals has been widely reported. A prime example is the insecticide Lindane, which

when absorbed into the body accumulates in the CNS and the brain and has been linked with cancer.[26] Therefore, the use of Lindane has been restricted in the United States and has been replaced by the far safer insecticide permethrin.[27] Although, more recently one case of permethrin poisoning was reported after application to damaged skin.[28] Other pesticides have been found to be genotoxic after topical exposure, including aminocarb, chlordane, DDT, and Dichlorous.[29] Chlorophenoxy herbicides cause a variety of systemic toxicities, including transient GI irritation and progressive mixed sensor motor peripheral neuropathy.[30] More recently, new guidelines have been proposed for the safety assessment of systemic toxicity caused by agrochemicals, to take into account the significant exposure after skin contact.[31] Agrochemicals such as parathion, malathion, and chlordane have been reported to persist in the skin up to 2 years after exposure.[32]

18.3.2 Antibiotics

18.3.2.1 Chloramphenicol

Oral administration of chloramphenicol may lead to aplastic anemia.[33] Marrow aplasia with a fatal outcome after topical application of chloramphenicol in eye ointment was described by Abrams et al.[34] This claim has been more recently disputed, and Walker and co-workers[35] concluded that topical chloramphenicol should not be considered to be a health hazard.

18.3.2.2 Clindamycin

Topical clindamycin is widely used in the treatment of acne vulgaris. Approximately 5% clindamycin hydrochloride is absorbed systemically.[36] The degree of absorption largely depends on the vehicle, ranging from 0.1 (acetone) to 14% (DMSO).[37] Several cases of topical clindamycin-associated diarrhea have been reported.[38] Pseudomembranous colitis is a side effect of systemic administration of clindamycin. A case of pseudomembranous colitis has been reported after topical administration by Milstone et al.[39] An updated overview is reported by Akhaven and Bershad.[40]

18.3.2.3 Gentamycin

Ototoxicity is a well-known toxic effect of systemic gentamycin administration. However, topical application to large thermal injuries of the skin has similarly caused ototoxic effects, ranging from mild to severe hearing loss, with an associated decrease of vestibular function.[41,42] Drake[43] described a woman who developed tinnitus each time she treated her paronychia with gentamycin sulfate cream 0.1%. Use of gentamycin eardrops may also be associated with ototoxic reactions.[44]

18.3.2.4 Neomycin

Just as ototoxicity is a well-known hazard of parenteral neomycin administration, so has deafness been reported after local treatment, including skin infections and burns,[45] application as an aerosol for inhalation, instillation into cavities,[46] irrigation of large wounds,[47] and use of neomycin containing eardrops.[48] Kellerhals[49] reported 13 cases of inner ear damage in which the use of eardrops containing neomycin and polymycin were incriminated. All cases had perforated tympanic membranes.

18.3.3 Antihistamines

18.3.3.1 Diphenylpyraline Hydrochloride

Diphenylpyraline hydrochloride is a histone H1 receptor agonist and has been used topically in Germany for the treatment of eczematous and other itching dermatoses. Symptomatic psychosis has been observed in 12 patients, 9 of which were children. The amounts of the active drug applied ranged from 225 to 1350 mg. The first symptoms of toxicity were psychomotor restlessness in all cases, usually within 24 h. Other symptoms included disorientation, and optic and acoustic hallucinations. All symptoms disappeared 4 days after discontinuation of the topical medication.[50] Recent studies have shown that diphenylpyraline hydrochloride acts as a dopamine transporter inhibitor similar to cocaine.[51]

18.3.3.2 Doxepin

Five percent topical doxepin has been introduced as an antipruritic. Percutaneous absorption frequently leads to clinical sedation.[52,53]

18.3.3.3 Promethazine

Block and Beysovec[54] reported a 16-month-old male treated with 2% promethazine cream for generalized eczema. The child showed abnormal behavior, loss of balance, inability to focus, irritability, drowsiness, and failure to recognize his mother. A diagnosis of promethazine toxicity through percutaneous absorption was made.

18.3.4 Antimicrobials

18.3.4.1 Boric Acid

The toxicity of this mildly bacteriostatic substance is reviewed in detail by Stewart et al.[55] The misuse of borates has been abandoned because of their limited therapeutic value and high toxicity, resulting in few new cases of borate intoxication.

18.3.4.2 Castellani's Solution

Castellani's solution (or paint) is an old medicament mainly used for the local treatment of fungal skin infections. It contains boric acid, fuchsin, resorcinol, water, phenol (90%), acetone, and spirit. Lundell and Nordman[56] reported a case in which two applications of Castellani's solution severely poisoned a 6-week-old boy who became cyanotic with 41% methemoglobin. Another case report states that hours after the application of Castellani's paint to the entire body surface (except the face) of a 6-week-old infant for severe seborrheic

dermatitis, the child became drowsy and had shallow breathing.[57] Castellani's solution can no longer be used for medication due to the critical status of its ingredients.[58]

18.3.4.3 Hexachlorophene

Since 1961, hexachorophene has been extensively used[59] mainly for reducing the incidence of staphylococcal infections among the newborn. In addition, it has been an ingredient of many medical preparations, cosmetics, and other consumer goods. Hexachlorophene readily penetrates damaged skin, and its absorption through intact skin has also been demonstrated.[60,61] In 1972, as a result of the accidental addition of 6.3% of hexachlorophene to baby talcum powder, 204 babies fell ill (convulsions, behavioral changes, and CNS depression) and 36 died from respiratory arrest.[62] This report was followed by animal experiments with hexachlorophene confirming that the drug is neurotoxic.[63] It has effects on motor, sensory, or cognitive function that are detectable using functional measures such as behavior. Furthermore, there is evidence that if exposure occurs during critical periods of development, many of the chlorinated hydrocarbons are developmental neurotoxicants.[63] Developmental neurotoxicity is frequently expressed as alterations in motor function or cognitive abilities or changes in the ontogeny of sensorimotor reflexes. Marzulli and Maibach[64] have placed in perspective lessons to be learned from its toxicity.

18.3.4.4 4-Homosulfanilamide

4-Homosulfanilamide (sulfamylon) is a topical sulfonamide used for the treatment of large burns. Sulfamylon is a carbonic anhydrase inhibitor and has caused hyperchloremic metabolic acidosis in patients with extensive burns treated topically, caused by percutaneous absorptions of the drug.[65] Reversible pulmonary complications and methemoglobinuria have also been reported.[66]

18.3.4.5 Povidone-Iodine

Povidone-iodine (Betadine) is a water-soluble iodine complex that retains the broad-range microbiocidal activity of iodine, without the undesirable effects of iodine tincture. However, toxicity still occurs from povidone-iodine percutaneously absorbed, mainly when it is used on large areas of burnt skin or on neonates. Further information can be found in the review by Postellon and Aronow.[67]

18.3.4.6 Phenol

In dilutions of 0.5–2%, phenol is sometimes prescribed as an antipruritic in topical medicaments and is used for phenol face peels. It is readily absorbed through the skin and has been shown to have a prolonged elimination due to extensive tissue distribution.[68,69] Phenol-induced ochronisis has been reported in patients, who for many years treated leg ulcers with wet dressings containing phenol.[70] Several case reports document fatal reactions to percutaneously absorbed phenol: by accidental spillage of phenol,[71] due to treatment of burns with a phenol-containing preparation,[72] and to the application of phenol to wounds.[73] A 1-day-old child died after application of 2% phenol to the umbilicus.[74] Several cases of sudden death and intra- or postoperative complications have been reported after phenol face peels.[75] Major cardiac arrhythmias were noted in 10 out of 43 patients during phenol face peels.[76] However, this is rather controversial and some authors feel that when the procedure is performed correctly phenol face peels are not potentially hazardous.[77]

18.3.4.7 Resorcinol

Resorcinol is used for its keratolytic properties in the treatment of acne vulgaris. It is also a constituent of the antifungal Castellani's solution. Formerly, leg ulcers were treated with external applications of resorcinol. It has an antithyroid activity similar to that of methyl thiouracil. Consequently, several cases of myxoedema caused by percutaneous absorption of resorcinol, especially from ulcerated surfaces, have been described.[78] Resorcinol, administered at high doses, can disrupt thyroid hormone synthesis and can produce goitrogenic effects.[79] However, risk-assessment analysis of these effects from "real-world" conditions suggests that human exposure to resorcinol is not expected to cause adverse effects on thyroid function after topical application.

Methemoglobinemia in children, caused by absorption of resorcinol applied to wounds, has also been reported.[80] Cunningham[81] reported many cases of infant toxicity, including, cyanosis, hemolytic anemia, and hemoglobinemia. In the literature, the author found seven cases of acute poisoning in babies as a consequence of topical resorcinol application and five fatalities were recorded. Although the use of resorcinol in young children and for leg ulcers should be avoided, topical resorcinol, when used for acne vulgaris, has been reported to be safe.[82]

18.3.4.8 Silver Sulfadiazine

Silver sulfadiazine is intended primarily for the control of pseudomonas infections in burns patients. Its relative freedom from side effects has contributed to its popularity. There have been reports of nephrotic syndrome, leucopenia, and granulocytosis following topical therapy.[83–85] Current evidence suggests a causal relationship of silver sulfadiazine with leukopenia, although the mechanism of this reaction is unknown. The drug presumably affects the white blood cells peripherally, but not the erythrocyte count. The sulfadiazine-induced leukopenia is at its nadir within 2–4 days of starting therapy, with the leucocyte count returning to normal within 2–3 days, and recovery is not affected by contination of therapy. Maitre et al.[86] have reported several cases of systemic silver accumulation in patients undergoing prolonged silver sulfadiazine treatment. It provokes hepatic, renal, and neurological tissue toxicity.[86]

18.3.5 Aromatic Amines

Aromatic amines are known to cause a wide range of systemic toxicities, from acute hepatotoxicity to carcinogenic effects.

4,4′-Methylenedianiline and 4,4′-methylene-bis-chloroaniline are two widely used aromatic amines employed in the manufacture of polyurethane foams, epoxy resins, and as curing agents in rubber manufacture. These two chemicals have been shown to be carcinogenic and mutagenic in a number of animal species, and induce liver damage in humans and rats.[87–89] Both chemicals have been detected in the urine of factory workers[90] and several authors have reported extensive absorption through human and rat skin *in vitro*.[91,92]

18.3.6 Arsenic

von Roemeling et al.[93] reported multifocal malignancies of the bowel and bladder in a psoriatic patient treated 20 years with topical Fowler's solution, indicating percutaneous absorption can also be carcinogenic. Dermal exposure to arsenic can cause cardiovascular diseases, developmental abnormalities, neurological disorders, hearing loss, various types of cancer, and diabetes.[94] However, the severity of the reaction is directly related to the chemical form of arsenic to which the individual is exposed.

18.3.7 Camphor

Camphor is a cyclic ketone of the hydroaromatic terpene group. It is an ingredient of a large number of over-the-counter topical remedies (with a camphor content of 1–20%), taken especially for symptomatic relief of "chest congestion" and "muscle aches." Camphor is readily absorbed from all sites of administration, including topical application. The compound is classified as a class IV chemical, i.e., a very toxic substance. Hundreds of cases of intoxications have been reported, usually after accidental ingestion by children[95] and usually from exposure to relatively small amounts.[96]

18.3.8 Cosmetic Agents

Cosmetic ingredients and fragrance materials are derived from a class of chemicals generally characterized by low toxicity. In a study by Di Giovanni et al.,[97] 3500 cosmetics consumers were questioned and of all the adverse effects caused by cosmetic use, only 4% of reactions were systemic (including headaches and nausea). Henna dye is used on nails, skin, and hair by married women in the Islamic community and consists of the dried leaves of *Lawsonia alba* (the coloring matter, lawsone, is a hydroxynaphthoquinone) and when mixed with *p*-phenylenediamine (to accelerate the fixing) over 20 cases of toxicity, some fatal, have been noted in Khartoum alone over a 2-year period. Initial symptoms are those of angioneurotic edema with massive edema of the face, lips, glottis, pharynx, neck, and bronchi and occur within hours of skin exposure. The symptoms may then progress on the second day to anuria, renal tubular necrosis, and acute renal failure with death occurring on the third day.[98] Whether this toxicity is due to *p*-phenylenediamine *per se* (probably grossly impure) or its toxicity is potentiated in its combination with henna is unknown. Systemic administration of the *p*-phenylenediamine leads to similar symptoms.[99]

Spencer and Bischoff[100] reported that after skin penetration musk ambrette (mainly used as a fragrance) causes the breakdown of cellular elements within the brain, spinal cord, and peripheral nerves. These types of effects were also reported for the fragrance acetyl ethyl tetrametyl tetralin.

Recently, it has been suggested in the literature that certain UV filters, a common cosmetic ingredient, represent a new class of endocrine active chemicals, for example, 3-benzylidene camphor.[101,102]

18.3.9 Crude Oil

Feuston et al.[103] have reported major systemic toxic effects after the dermal application of crude oils to rats. The major effects included: reduction in body weight gain, increases in absolute and relative liver and thymus weight. Red blood count, hemoglobin, hematocrit, and platelet count were all affected. These effects were related to concentrations of polycyclic aromatic compounds found in the crude oil.

18.3.10 Dimethyl Sulfoxide

The toxicology of topical dimethyl sulfoxide (DMSO) has been investigated by Kligman.[104] In this study, except for the appearance of cutaneous signs as erythema, scaling, contact urticaria, stinging, and burning sensations, DMSO was tolerated well by all but two individuals, who developed systemic symptoms. In one, a toxic reaction developed that was characterized by a diffuse erythematous and scaling rash accompanied by severe abdominal cramps; the other had a similar rash, nausea, chills, and chest pains. These signs, however, abated in spite of continued administration of the drug. One fatality due to a hypersensitivity reaction has been alleged.[105]

18.3.11 Dinitrochlorobenzene

Dinitrochlorobenzene (DNCB), a potent contact allergen, has been used for the treatment of recalcitrant alopecia areata. Today, however, its use has been discouraged because of suspicion that DNCB may be mutagenic and the fact that it is absorbed in substantial amounts through the skin, with about 50% of an applied dose recoverable in the urine.[106] Possible systemic reactions to DNCB have been reported.[107] For example, a 25-year-old man treated with 0.1% DNCB (daily for 2 months) after prior sensitization experienced generalized urticaria, pruritus, and dyspepsia.

18.3.12 Ethanol

Twenty-eight children with ethanol toxicity from percutaneous absorption were described by Gimenez et al.[108] following a popular procedure where ethanol-soaked cloths are applied to the abdomen of babies as a "home-remedy" for the treatment of disturbances of the GI tract, or because of crying, excitability, and irritability. Ethanol-soaked cloths had been applied under rubber panties, and the number of applications varied from one to three (40 mL/application). All 28 children showed some degree of CNS depression, 24 showed miosis,

15 hypoglycemia, 5 convulsions, 5 respiratory depression, and 2 died. Of the two who died, one was autopsied and the findings were consistent with ethanol toxicity. Topically applied ethanol in tar gel and beer-containing shampoo has caused Antabuse effects in patients on disulfiram for alcoholism, through percutaneous absorption.[109,110]

18.3.13 Fumaric Acid Monoethyl Ester

The effect of systemically or topically administered fumaric acid monoethyl ester (ethyl fumarate) on psoriasis was studied by Dubiel and Happle[111] in six patients. Two patients who had been treated dermally developed symptoms of renal toxicity.

18.3.14 Pesticides

18.3.14.1 Lindane

Lindane, the γ-isomer of benzene hexachloride, is widely used in the treatment of scabies and pediculosis. The percutaneous absorption of the drug has been widely documented,[112,113] as has toxicity from excessive topical therapeutic application of Lindane.[114] The issue of possible toxic reactions to a single therapeutic application of Lindane, notably CNS toxicity, has not been completely settled. Lindane absorption through human skin is more rapid than through animal skin. It is relatively slowly metabolized, meaning a possible accumulation and slow removal from the blood and brain.[115] Therefore, over the past few years the safer alternative, permethrin, has evolved as the standard care for scabies.

18.3.14.2 Diethyltoluamide

N,*N*-diethyl-*m*-toluamide (DEET) has been used as an effective insect repellent for nearly 50 years. It is commercially available in various topical forms containing between 10 and 95% DEET. Although DEET has an overall low incidence of toxicity, prolonged use in children has been discouraged because of reports of toxic encephalopathy and death.[116] Although most reports of CNS toxicity have been in children, adults and fetuses may also be at risk. Long-term occupational exposure has led to episodes of confusion, depression, insomnia, and muscle cramps.[117] Schaefer and Peters[118] reported a 4-year-old boy with mental retardation, impaired sensorimotor coordination, and craniofacial dysmorphology, whose mother applied a lotion containing 25% DEET daily to her arms and legs throughout her pregnancy. Other systemic toxicities reported include seizures, cardiovascular toxicity, with a few cases of death due to extensive skin absorption.[119]

18.3.14.3 Malathion

The detailed toxicology of malathion is dealt with by Haddad.[59] Malathion is used in the treatment of lice (0.5% solution being customary). Used in this way, it is generally safe. Ramu et al.[120] reported four children with toxicity following hair washing with 50% malathion in xylene for the purpose of louse control. Tos-Luty et al.[121] conducted dermal toxicity studies in rats and concluded that higher doses of malathion applied dermally exerted a damaging effect on the intracellular structure of the liver, kidney, heart, and lungs.

18.3.14.4 Paraquat

The herbicide paraquat has been shown to cause genotoxicity in the bone marrow of rats after dermal application.[122] It has also been shown to be genotoxic and cytotoxic to germ cells in the male rat.[123]

18.3.14.5 Combination Effects

Some of these common pesticides have been studied in combination.[124] These authors concluded that real-life doses of DEET, permethrin, and malathion, alone or in combination, produce no overt signs of neurotoxicity, but do induce significant neurobehavioral deficits and neuronal degeneration in the brain of rats.

18.3.15 Local Anesthetics

18.3.15.1 Benzocaine

Methemoglobinemia has been reported following the topical application of benzocaine (ethyl aminobenzoate) to both skin and mucous membranes, with most cases occurring in infants.[125–127] However, toxicity is uncommon.

18.3.15.2 Lidocaine

Lidocaine hydrochloride is widely used for topical and local injection anesthesia. Serum lidocaine concentrations, higher than 6 μg/mL, are associated with toxicity,[128] whose signs include CNS stimulation followed by depression and later inhibition of cardiovascular function. Systemic toxicity from lidocaine applied to the oral cavity in two children has been described.[129,130]

18.3.16 Mercurials

Mercury is a toxic and hazardous metal and its mechanisms of toxicity are comprehensively dealt with by Aronow.[131] With few exceptions, the use of mercury in medicine is considered to be outdated. However, mercury may still be present in many drugs, even in over-the-counter formulations. Metallic mercury is readily absorbed through intact skin, as is ammoniated mercury chloride in psoriatic patients.[132] Young[133] examined 70 psoriatic patients treated with an ointment containing ammoniated mercury. Symptoms and signs of mercurial poisoning could be detected in 33 patients. Nephrotic syndrome has been reported after ammoniated mercury-containing ointment application.[134,135] There have been two case reports of children who died following the treatment of an omphalocele with merbromin (an organic mercurial antiseptic).[136]

18.3.17 Monobenzone

Monobenzone (monobenzyl ether of hydroquinone) is used topically by patients with extensive vitiligo to depigment

their remaining normally pigmented skin. In 11 patients with vitiligo, on monobenzone therapy, conjunctival melanosis and pingueculae was acquired.[137]

18.3.18 Monochloroacetic Acid

Monochloroacetic acid has caused many fatal occupational accidents via skin exposure.[138] These authors concluded that the severe toxicity was probably a consequence of rapid absorption causing hepatocellular injuries, renal dysfunction, dysglyconeogenesis, and perturbation of ammonia metabolism. Monochloroacetic acid is thought to enter the tricarboxylic acid (TCA) cycle and inhibit aconitase.

18.3.19 2-Naphthol

2-Naphthol (β-naphthol) is used in peeling pastes for the treatment of acne, and between 5 and 10% of a cutaneous dose has been recovered from the urine of subjects.[139] Extensive applications of 2-naphthol ointments have been responsible for systemic side effects, including vomiting and death.[140] Hemels[139] concludes that 2-naphthol-containing pastes should be applied only for short periods of time and to a limited area not exceeding 150 cm^2.

18.3.20 Podophyllum

The toxicity of podophyllum was reviewed by Cassidy et al.[141] Although there have been a significant number of case reports describing serious neurological illness or death following the application of podophyllum, these are generally related to its use in widespread dermal lesions.[142,143] Systemic symptoms after dermal exposure include, thrombocytopenia, leucopenia, abnormal liver function, sensory ataxia, and neurological effects. A cause of suspected teratogenicity (simian crease on left hand and preauricular skin tag) has also been reported after topical podophyllum resin treatment.[144]

18.3.21 Retinoic Acid

Antiacne creams have been shown to cause systemic toxic effects. For example, retinoic acid is a known teratogen and is found in certain formulations.[145]

18.3.22 Salicylic Acid

The general toxicology and percutaneous absorption of salicylates is reviewed by Proudfoot.[146] Salicylic acid (SA) is widely used in dermatology as a topical application for its keratolytic properties, and salicylate poisoning after topical use has been reported. An unpublished review by the U.S. Department of Health, Education and Welfare, quoted by Rasmussen,[10] revealed 13 deaths associated with the widespread use of SA preparations, 10 in children. von Weiss and Lever[147] reported 13 deaths resulting from intoxication with SA following application to the skin, reported in literature up to 1964, and several nonfatal intoxications. The most dramatic account is that of two plantation workers in the Solomon Islands, who were painted twice daily with an alcoholic solution of 20% SA involving about 50% of the body. The victims were comatose after 6 h and dead within 28 h.[148] A case of SA toxicity leading to coma in an adult patient with psoriasis, who had been treated with 20% SA in petrolatum, was also described by Treguer et al.[149] Metabolic acidosis has also been reported after percutaenous absorption of SA.[150,151] Chronic SA exposure can also cause systemic inflammatory response syndrome.[152]

18.3.23 Selenium Sulfide

Ransone et al.[153] reported a case of systemic selenium toxicity in a woman who had been shampooing her hair two or three times weekly for 8 months with a selenium sulfide suspension.

18.3.24 Silver Nitrate

Ternberg and Luce[154] observed fatal methemoglobinemia in a 3-year-old girl suffering from extensive burns, who was treated with silver nitrate solution. Owing to the hypotonicity of the silver nitrate dressings, hyponatremia, hypokalemia, and hyperchloremia may develop, especially in children.[155] Excessive use of silver-containing drugs has led to local and systemic argyria and to renal damage involving the glomeruli with proteinuria.[156,157]

18.3.25 Steroids

18.3.25.1 Corticosteroids

Topically applied glucocorticosteroids are absorbed through the skin,[158] resulting in sufficient quantities in the systemic to replace endogenous production. Systemic side effects of topical corticosteroids occur more frequently in children than adults and in patients with liver disease due to reduced metabolism of the drug.[159,160] The two main causes of systemic side effects are hypercorticism leading to an iatrogenic Cushing's syndrome and suppression of the hypothalamic-pituitary-adrenal axis.[161] In rat's topical application of several corticosteroids caused body weight gain suppression, total cholesterol and triglycerides were increased and the lymphatic tissue was atopic. In addition, several corticosteroids caused adrenal and renal lesions.[162]

18.3.25.2 Sex Hormones

Topical application of estrogen-containing preparations leads to resorption of these hormones and therefore systemic estrogenic effects. Beas et al.[163] reported on seven children with pseudoprecocious puberty due to an ointment containing estrogens. The most important clinical signs were intense pigmentation of mammillary areola, linea alba of the abdomen and the genitals, mammary enlargement, and the presence of pubic hair. Three female patients also had vaginal discharge and bleeding. After discontinuation of the drug, all symptoms progressively disappeared in every

patient. Pseudoprecocious puberty has also been observed in young girls after contact with hair lotions and other substances containing estrogens.[164] Gynecomastia has been reported in young boys and men.[165–167]

18.3.26 Miscellaneous

There are many other examples of systemic toxicity caused by absorption through the skin. For example, exposure to acrylamide dust in polymer factories, causing a chronic disease of the nervous system.[168] Skin exposure to ethylene glycol dinitrate during dynamite production results in toxic effects after only a few minutes.[169] Carbon tetrachloride and 2-chloroethanol cause hepatotoxicity and hepatocarcinogenicity.[170] Glycol ethers, in particular ethylene glycol monoethylene ether are teratogenic and cause menstral disorders in women.[171] Mint[172] showed that repeated dermal exposure of rats *in vivo* to dibutyl phthalate caused significant hepatic peroxisome proliferation within 14 days.

Transdermal systems contain an excess amount of drug to maintain the needed concentration gradient for drug delivery. Upon removal, patches still retain a substantial amount of active drug,[173] increasing the risk of toxicity if applied to the skin of an infant or young child, and emphasizing the need for proper use and disposal of transdermal drug-delivery systems.

Monoethanolamine, diethanolamine, triethanolamine (TEA) are industrial chemicals, and the principle route of exposure is through the skin. Systemic effects after 2-year TEA administration included hyperplasia of the renal tunular epithelium and small microscopic adenomas and nonneoplastic changes in the liver.[174]

18.4 COMMENT

This chapter summarizes literature citations and the basic aspects of percutaneous penetration to alert the reader to the potential for systemic toxicity from topical exposure. From the information provided in this chapter, the reader can clearly see that systemic toxicities are important considerations for a diverse group of compounds after skin exposure. The severity of these systemic reactions is often worse in young children/infants or in patients with impaired barrier function (i.e., due to increased absorption).

Demonstrating causality (rather than association) requires careful documentation. Combining knowledge of the inherent molecular and animal toxicology, cutaneous penetration, and metabolism with the adverse human reaction literature permits a more precise determination of causality. The above data focus the need for controlled studies on the toxicity of chemicals, which come into contact with the skin, either accidentally or deliberately. There are many other texts emphasizing current approaches and technology.[1–3,175]

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19 Concepts in Molecular Dermatotoxicology

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19.1 INTRODUCTION

The skin is a major interface between the environment and the body. Many investigations of its barrier functions by means of pharmacokinetic methods have been performed and have demonstrated that the skin is a very effective barrier against most potential environmental hazards. However, the awareness of multiple functional activities of this organ such as its capability (1) to mediate enzyme-dependent xenobiotica metabolism, (2) to express specific transporter proteins for influx as well as for efflux into or out of its cells, (3) of multiple activities as an immunocompetent organ including the antigen-presenting Langerhans cells, and (4) of interactions of these cells for the protection of the body against the hazards of ultraviolet (UV) radiation has resulted in new and additional concepts about how the skin works as a barrier organ [1,2]. In addition, new technologies such as quantitative real-time PCR or the gene-array technique, which can be used for human *in vivo* studies, have offered superb possibilities to analyze the multiple capabilities of this complex organ system. These developments have also led to new avenues of research in dermatotoxicology and may provide powerful instruments for dermatotoxicological risk assessments.

To illustrate this exciting development, the molecular mechanisms of UV as well as infrared radiation-induced biological effects will be discussed, followed by the presentation of the cutaneous armentarium of xenobiotica metabolizing enzymes and recent findings about cutaneous transporter proteins. Finally, three examples of toxic events by small molecular weight compounds—cutaneous chemocarcinogenesis, drug allergy and allergic contact dermatitis (ACD)—and the role of xenobiotica metabolism will be outlined.

19.2 MOLECULAR MECHANISMS OF UV AND INFRARED RADIATION-INDUCED BIOLOGICAL EFFECTS

As a result of increased leisure time, the growing popularity of staying outdoors, holidaying in the sun as well as the use of sunbeds, human skin is increasingly exposed to shortwave (290–320 nm; UVB) and longwave (320–400 nm; UVA) UV radiation. UV radiation is well known to exert a variety of

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deleterious effects on human skin [3]. Chronic and possibly also acute exposure to UV radiation increases the risk of developing skin cancer including basal cell carcinoma as well as malignant melanoma. UV radiation causes premature skin ageing, which is characterized by generalized wrinkling, dry and thin appearance, and seborrhoeic keratoses [4]. There are also several specific diseases, which are triggered or exacerbated by UV radiation exposure, such as phototoxic or photoallergic reactions, autoimmune diseases including lupus erythematosus and idiopathic photodermatoses, for example, polymorphous light eruption or solar urticaria. In addition to UV radiation, exposure to near infrared radiation from sunlight or artificial irradiation devices has recently been found to cause skin damage in a manner similar to that observed after UV irradiation [5].

19.3 MOLECULAR BASIS OF THE UVB RESPONSE: NUCLEAR DNA DAMAGE, MEMBRANE EFFECTS, CYTOPLASMIC SENSORS AND MORE

For UVB radiation-induced signal transduction, the generation of photoproducts within the DNA of epidermal keratinocytes is thought to be of critical importance. Among the DNA lesions induced by UVB radiation, cyclobutane pyrimidine dimers predominate. Dimer formation is crucial for the initiation of skin cancer, because it is closely linked to the generation of mutations in tumor suppressor genes, expressed in UV-induced skin tumors [6]. There is, however, also a growing evidence that dimers contribute to photocarcinogenesis by the suppression of the skin's immune system, allowing the transformed cells to grow unimpeded [7]. This information has prompted the development of liposomes, which contain functionally active, dimer-specific DNA repair enzymes and which can be topically applied to human skin to achieve dimer repair and prevent UVB radiation-induced immunosuppressive effects. The effectiveness of this approach has first been demonstrated in animal models, and more recently in human skin [8]. In this study, topical application of photolyase-containing liposomes to UVB-irradiated human skin and subsequent exposure to photoreactivating light has been found to partially remove the UVB radiation-induced dimers in epidermal keratinocytes and to completely prevent the UVB radiation-induced immunosuppressive effects. Also, in a consecutive placebo-controlled multicenter trial, it has been demonstrated that daily application of a DNA repair enzyme containing liposome lotion is effective in preventing the generation of precancerous and cancerous lesions in patients with the DNA repair deficiency syndrome xeroderma pigmentosum [9].

In addition to DNA damage, membrane effects have been shown to play a crucial role in UVB radiation-induced signal transduction [10]. UVB radiation-induced biological effects in keratinocytes such as gene expression or apoptosis (which occurs at higher UVB doses) can be prevented by removal of UVB radiation-induced dimer formation. This inhibition, however, is only partial. Similarly, partial inhibition can be achieved, if the UVB radiation-induced clustering of growth factor receptors on the surface of the plasma membrane of keratinocytes, rather than nuclear DNA damage, is inhibited. However, complete inhibition of UVB-induced effects was found to require prevention of both DNA damage and receptor clustering [11]. The precise biochemical nature of UVB radiation-induced membrane effects is not yet clear and the relevant chromophore(s) unknown. It has been established, however, that receptor clustering is followed by a well-defined cascade of signalling steps that eventually lead to MAP kinase (MAPK) activation, transcription factor activation, and increased gene expression [10]. In this regard, it is of interest that a similar, if not identical, signalling pathway may be triggered in unirradiated cells that are exposed to hyperosmotic stress, indicating the possibility that alterations in cell volume homeostasis may be of relevance to UVB radiation-induced signalling. Accordingly, recent studies have demonstrated that the presence of osmolyte transport systems in human epidermal keratinocytes and osmolyte loading of keratinocytes prevents UVB radiation-induced biological effects such as apoptosis or gene expression (Krutmann J., Grether-Beck S., Schade N., Esser C., unpublished observations). The exact level at which osmolytes interfere with UVB radiation-induced signalling is currently not known.

It should be noted that UVB radiation, in addition to DNA, can be absorbed by several other molecules, some of which are present in the cytoplasm of cells as well. In this regard we have recently shown that part of the UVB response involves cytoplasmic tryptophan as a chromophore, and through the photochemical concepts in molecular dermatotoxicology, generation of the arylhydrocarbon receptor (AhR) ligand 6-formyindolo[3,2]-b-carbazole initiates signalling events, which are transferred to the nucleus and the cell membrane via activation of the cytoplasmic AhR [89].

19.4 THE UVA RESPONSE: FROM CELL MEMBRANE LIPIDS TO MITOCHONDRIA

There is an increasing evidence that similar to UVB radiation, UVA radiation plays a pivotal role in the pathogenesis of skin cancer, in photodermatosis such as polymorphous light eruption and extrinsic (1/4 photo) ageing of human skin. In recent years, substantial progress has been made in understanding the mechanisms through which UVA radiation induces gene expression in human skin cells [12]. In keratinocytes, UVA radiation-induced gene expression was found to involve the formation of second-messenger ceramide from cell membrane sphingomyelin through a photobiological mechanism that is mediated by the generation of singlet oxygen and is capable of hydrolyzing the cell membrane sphingomyelin through a nonenzymatic mechanism [13]. Second-messenger ceramide, without the induction of apoptosis, subsequently acts on mitochondria and causes the release of mitochondria-derived cytochrome C into the cytoplasm [14]. Within the cytoplasm, a redox interaction between cytochrome C and a transcription factor, termed AP-2, occurs, which leads to the oxidation

of AP-2 and reduction of cytochrome C. Oxidized AP-2 has a greatly increased capacity to bind to AP-2 consensus binding sites within the promoter region of UVA-responsive genes and has been shown to be responsible for increased transcriptional expression of UVA radiation-inducible genes in human keratinocytes such as ICAM-1 [15]. UVA radiation-induced signalling thus involves the generation of reactive oxygen species at a very early stage, that is, during the generation of second-messenger ceramide, and at a later stage, that is, during the oxidation of AP-2. This knowledge has been used in further studies to modulate the UVA radiation-induced gene expression by means of various antioxidants.

Further analysis of the very early, that is, the initial signalling events responsible for UVA radiation-induced gene expression, identified signal-transducing microdomains (rafts) within the keratinocyte membrane as critical targets for UVA radiation. Accordingly, enrichment of cell membranes with cholesterol rendered keratinocytes completely unresponsive to UVA radiation (Krutmann J., Grether-Beck S., unpublished observations). These observations indicate that cholesterol and cholesterol-like molecules may serve a previously unrecognized role in photoprotection of human skin.

Similar to UVB, UVA radiation-induced signalling in keratinocytes can be effectively prevented by treatment of cells with osmolytes such as taurine. In addition, preliminary evidence suggests that osmolytes interfere with UVA radiation-induced signalling at a very early stage, for example, by preventing the UVA radiation-induced ceramide formation (Krutmann J., Grether-Beck S., unpublished observation). The exact mechanisms by which osmolytes stabilize cell membrane lipids or rafts are currently under investigation.

19.5 INFRARED RADIATION-INDUCED SIGNALLING IN HUMAN SKIN CELLS

Human skin is exposed to infrared radiation (760 nm to 1 mm) from natural as well as artificial sources that are increasingly used for cosmetic or medical purposes [5]. Epidemiological data and clinical observations indicate that infrared radiation can damage the human skin. In particular, infrared radiation, similar to UV radiation, seems to be involved in photoageing and possibly also in photocarcinogenesis. Recent studies indicate that the basic molecular processes such as signal transduction and gene expression, triggered by exposure to infrared radiation, differ to some extent from those induced by UV radiation. Accordingly, infrared radiation, but not UV radiation-induced upregulation of the mRNA and protein expression of matrix metalloproteinase-1 in human skin fibroblasts, required extracellular signal-regulated kinase 1/2 (ERK1/2) activation [16]. In addition to ERK1/2, infrared radiation has also been shown to activate the p38-MAPK (mitogen-activated protein kinase) signalling pathway. Because MAPKs are involved in transcriptional regulation of a multitude of genes, it is very likely that infrared radiation is able to influence the expression of several genes via activation of MAPK signalling pathways. Taken together, these observations indicate that infrared radiation is capable of eliciting a molecular response in human skin cells similar to, but not identical, the response induced by UV radiation. We have, therefore, recently proposed the term "infrared response" to emphasize the ability of infrared radiation to influence cellular functions such as signal transduction and gene expression specifically [5].

The photophysical and photochemical mechanisms that mediate the before-mentioned molecular effects and biological consequences of infrared radiation are largely unknown. Recent studies from our laboratory suggest that oxidative processes, which are initiated in the mitochondrial electron transport chain of infrared-irradiated human cells, are critically involved in infrared radiation-induced gene expression. These studies may also allow the development of photoprotective strategies against unwanted infrared radiation effects [90].

Although the majority of skin cancers are linked to exposures to UV irradiation, several chemicals were found to be able to induce skin carcinogenesis, and chimney sweep-induced skin cancer was the first reported chemically induced squamous skin tumor. In addition, cutaneous chemocarcinogenesis has become a classical model to study the processes of carcinogenesis on a molecular level. Most of the chemicals are small molecular weight compounds, which must be metabolized in most cases to highly reactive species to be the ultimate carcinogen [17]. The xenobiotica metabolizing enzymes, which are involved in this process, have been found to be present in the skin as well as transporter proteins for the influx and efflux of those compounds [18–20]. In particular, techniques of molecular dermatotoxicology have increased our knowledge about these proteins and their genes and allowed new possibilities to study this metabolism in extrahepatic organs such as the skin [21].

19.6 METABOLISM AND TRANSPORT IN HUMAN SKIN

For many years, the skin was thought to be a passive structural barrier between the body and the environment. We now know that the skin is indeed an active site of diverse types of metabolic activities involving an array of metabolizing enzymes. Recent research results demonstrate that (1) keratinocytes in human skin express various transport-associated and detoxifying metabolic enzymes, and (2) they are, therefore, capable of providing active uptake, biotransformation, and antitransport of different xenobiotics such as drugs, solvents, and carcinogens.

Besides the role of the stratum corneum as the most critical structure for epidermal barrier function, there is increasing evidence indicating that xenobiotic metabolizing enzymes and transport proteins function as the second biochemical barrier of the skin [22]. The metabolic interaction between small molecular weight compounds and molecular targets in the cell can at least be in these phases. After penetration, the xenobiotics are first chemically activated or inactivated by oxidative reactions [23]. The most important family of enzymes involved in these reactions is the family of

cytochrome P450 enzymes (CYP). Normal human epidermal keratinocytes and dermal fibroblasts reveal the constitutive expression of CYP1A1, 1B1, 2B6, 2E1, and 3A5 in the cytoplasm of epidermal keratinocytes and show the expression of CYP3A4 after incubation with drugs like dexamethasone [18,19]. Expression of the retinoic acid metabolizing CYP26AI is restricted to keratinocytes of the basal layer [24]. The expression of multiple CYP enzymes has also been shown in murine skin [25,26]. In addition to their detoxifying functions, these CYP enzymes may be involved in allergic reactions to substances of low molecular weight, for example, drug allergy or ACD as well as chemocarcinogenesis.

Efflux-transport associated proteins, such as P-glycoprotein (MDR1) and multidrug resistance-associated proteins (MRPs), are overexpressed in drug-resistant cell lines and human tumors from various histological origins, including malignant melanoma [27,28]. Several studies have been performed to investigate the expression, regulation, and specific substrates of these transport proteins in human skin cells [19,29].

Real-time polymerase chain reaction (RT-PCR) analysis of normal human epidermal keratinocytes (NHEK) revealed constitutive expression of MRP1 as well as MRP3–6 but was negative for MDR1 and MRP2. Expression of MDR1 was seen on the mRNA level after induction with dexamethasone. RT-PCR results were confirmed by immunoblots, which demonstrated the constitutive expression of MRP1, 3, and 5 as well as expression of MDR1 after induction with dexamethasone. Immunohistological analysis of healthy human skin showed positive staining for MRP1 in the cell membrane of epidermal keratinocytes [19]. Functional activity of MRPs in skin cells such as NEHK and dermal fibroblasts could be demonstrated *in vitro* using the calcein acetoxymethyl (AM) ester assay [30]. Other studies showed that mutations in the MRP6 gene cause pseudoxanthoma elasticum (PXE), a connective tissue disorder affecting the elastic structures in the body including the skin, although the exact pathomechanisms of PXE are not completely understood [31].

As regulatory mechanisms for these transporters in skin cells were unknown, influence of inflammatory cytokines on the expression of MRPs in these cells was analyzed [32]. Stimulation of NHEK and human dermal fibroblasts with interleukin-6 (IL-6), in combination with its soluble α-receptor (sIL-6R), or oncostatin M (OSM), for 24–72 h resulted in an upregulation of MRP expression and activity, which could be shown on the RNA level and by the use of efflux transport assays. Both cytokines induced a strong activation of signal transducer and activator of transcription STAT1 and STAT3 as well as the MAPK Erk1/2. OSM additionally activated protein kinase B strongly. Using the MAPK/extracellular signal-regulated kinase, kinase 1-specific inhibitor U0126, a stimulatory effect of MAPK on MRP gene expression could be excluded. Inhibition of the phosphatidylinositol 3-kinase, however, indicated that this pathway might be involved in OSM-mediated upregulation of MRP4 in dermal fibroblasts [32]. Several inflammatory skin diseases show an enhanced expression of IL-6-type cytokines. Corresponding to *in vitro* data, upregulation of MRP expression was found in lesional skin taken from patients with psoriasis and lichen planus [32].

Current studies focus on the specific substrates of the MRP-transporter expressed in the skin cells. Active transport of retinoids, which are known to have not only a pharmacological but also a physiological role in human skin and skin tumors, could be demonstrated. Treatment of cells with IL-6 and sIL-6R has increased the active efflux transport of all-trans [20-methyl-3H] retinoic acid in NHEK in comparison with transport measured in the absence of this cytokine [32]. Specific MRP-inhibitors like indometacin are able to inhibit this transport (Heise R., Rodriguez F., Neis M., Marquardt Y., Joussen S., Wosnitza M., Merk H. F., Baron J. M., 2006).

Influx-transport proteins, such as the organic anion transporting polypeptide (oatp1), were first identified on the molecular level in rats as a multispecific and sodium ion-independent transporter for various organic anions, including bile acids, conjugated metabolites, and xenobiotics. Subsequently, rat oatp2 and 3 were isolated as homologues of oatp1 and were shown to be present in various tissues and to transport anionic and neutral compounds. As they are structurally very closely related, they are considered to form a single protein family—the OATP family. In humans, OATP-A was first cloned from human liver as a homologue of rat oatp1. Tamai et al. [33] identified three novel human transporters (OATP-B, -D, and -E), structurally belonging to the organic anion transporting polypeptide (OATP) family. These OATPs exhibit a broad, and to a certain degree, overlapping substrate specificity and transport a wide range of more bulky organic compounds including steroid conjugates, cardiac glycosides, endothelin-antagonists, and certain toxins [33]. Studies by Schiffer et al. [20] were able to show a constitutive expression of OATP-B, -D, and -E in normal epidermal keratinocytes using RT-PCR and Northern blot analysis, as well as in human skin tissue shown by tissue blot hybridization and immunostaining. Expression of OATP-A and -C were not detected in any of the keratinocyte samples. Even though the substrate specificity of the OATP isoforms were only partially known until now, these findings gave a strong evidence that the uptake of large organic cations like drugs in keratinocytes is an active transport process mediated by members of the OATP family.

Interaction and synergism between CYP-mediated metabolism and active transmembrane transport occurs, if metabolites produced by the CYP enzymes are better substrates for transport proteins than the parent drug, or if the efflux transporter prolongs the duration of absorption by necessitating the repeated entry of the drug into the skin cell. This process increases the exposure of the substrate to the CYP enzyme and could, therefore, prevent kinetic saturation of this protein [18,34]. Resulting drug metabolites often have specific functions on gene expression in skin cells, which could be demonstrated for the 4-oxo metabolites of retinoic acid [35]. Interaction of the influx proteins of the OATP family and the efflux proteins of the MRP family has recently been

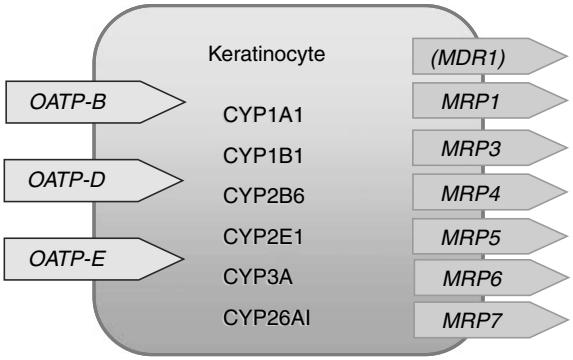


FIGURE 19.1 Transport and metabolism of xenobiotics in human skin cells [19,20]. Reprint of Merk, H.F., Baron, J.M, Heise, R., Fritsche, E., Schroeder, P., Abel, J., Krutmann, J., *Exp. Dermatol*., 15, 692–794, 2006. With permission.

described as a physiological mechanism for the transport of xenobiotics and has been named vectorial transport [36].

In conclusion, these studies showed that skin cells express various metabolizing and transport-associated proteins. Currently, it is presumed that in skin cells MRP transport proteins (phase III)—which play a major role in efflux of endogenous and exogenous compounds including leucotrienes and prostaglandins [37,38]—directly interact with the influx transport proteins such as OATPs (phase 0) and metabolizing enzymes such as CYPs (phase I and II) (Figure 19.1).

19.7 MECHANISM OF CHEMICAL CARCINOGENESIS

Occupational exposures to various chemical and topical applications of dermal medicinal preparations like coal tar have been considered as sources for skin cancer in humans. There is a broad group of environmental chemical agents known to produce skin cancer in animals; however, the contribution of such agents to human skin cancer is still unclear [39].

For skin carcinogenesis, it is generally believed that originating from a single mutated cell, the cancer develops over a series of preneoplastic and premalignant states. On the basis of animal studies, the multistage model of carcinogenesis has been developed. This operational model involves stepwise accumulation of several genetic changes leading to outgrowth of the malignant tumors. There is evidence that the stages of initiation, promotion, and progression involve qualitatively different kinds of molecular events. Initiation is an irreversible event, which occurs after a single treatment of mouse skin with a carcinogen. Tumor promoters do not act mutagenic by themselves, but promote the selection of initiated cells and induce the outgrowth of premalignant papillomas. Tumor progression is characterized by conversion of premalignant papillomas into carcinomas, and it has been suggested that additional mutational events are involved in the processes of conversion and progression. As the multistage model is intensely reviewed [40–42], we will summarize here the new findings in the field of chemically induced skin carcinogenesis.

An important step in skin carcinogenesis is tumor initiation that occurs by the interaction of a reactive chemical with DNA in the target cells. This interaction results in an irreversible alteration of genes involved in the regulation of terminal differentiation. Most of the chemicals with initiating activity have to be activated before inducing the DNA damage, and human and animal skins express the respective activating system, which catalyzes xenobiotic metabolism (see previous paragraphs in this review).

In experimental skin carcinogenesis, classical initiators are polycyclic hydrocarbons, such as benzo(a)pyrene (BaP) and 7,12-dimethylbenzo(a)anthracene (DMBA), which can be activated by the constitutively expressed cytochrome P4501A1 (CYP1A1) and cytochrome P450 1B1 (CYP1B1) [43]. An important function during the metabolic activation of polycyclic aromatic hydrocarbons has the aryl hydrocarbon receptor (AhR). This ligand-activated transcription factor is involved in the regulation of several phase 1 and phase 2 enzymes. Among the many AhR ligands, TCDD has been found to bind to the AhR with high affinity, and all the effects caused by this compound are mediated by the AhR. After ligand binding, the cytosolic AhR translocates to the nucleus and forms a heterodimer with ARNT. The AhR/ARNT dimer then binds to specific DNA sequences termed xenobiotic responsive element (XRE) and interacts with the transcriptional machinery. DNA microarray analyses have revealed that more than 300 genes are up- or down-regulated by this pathway including genes coding for phase 1 and phase 2 enzymes like CYP1A1, CYP1A2 CYP1B1, UGT 1A5 GSTYA and MNOR, respectively [44]. Recently, a AhR-related gene has been cloned and termed AhR repressor (AhRR). The AhRR is a heterodimer with ARNT and recognizes XRE, but functions as a transcriptional repressor [45]. AhR, AhRR, and their common partner ARNT are expressed in dermal and epidermal cells. The importance of the AhR in skin carcinogenesis could be demonstrated in AhR-deficient animals. Mice lacking in AhR are resistant toward BaP-induced skin tumorigenesis [46]. Besides its function in regulating the xenobiotic metabolism, the AhR seems to be functional in regulation of normal skin homeostasis. Skin of AhR-deficient mice exhibits interfollicular and follicular epidermal hyperplasia with hyperkeratosis and acanthosis associated with marked dermal fibrosis [47]. The involvement of AhR in regulation of skin homeostasis is supported by a recent study, in which transgenic mice with constitutive active AhR in keratinocytes were generated [48]. At birth, the transgenic animals were normal, but they developed severe skin lesions with itching, postnatally. The skin lesions were accompanied by inflammation and immunological imbalances and resembled typical atopic dermatitis. The inflammation-related genes, keratins 1, 6, and 16, the chemokine CCl 20, and IL-18 were strongly upregulated, reflecting the proinflammatory state of the skin of the transgenic animals. Based on these findings, the possibility has emerged for the authors that blocking the AhR signal may help to relieve the allergic symptoms, as many xenobiotics like P(a)H can exacerbate allergic reactions via the AhR pathway.

Metabolic activation and DNA binding of carcinogens in skin can produce successive accumulation of mutations, which can activate oncogenes or inactivate tumor suppressor genes. The p53 tumor suppressor gene is the most frequently mutated gene in humans [49] and in experimental models [50]. The mutations resulted in loss of function and are thought to play a causative role in tumorigenesis [49]; hence, p53-deficient animals develop a variety of spontaneous tumors such as lymphomas, osteosarcomas, and soft tissue sarcomas [51]. Although p53 mutations occur in skin cancer especially after UV radiation [41], it is not clear whether the loss of p53 function is critical in chemically induced skin carcinogenesis. After subjecting p53-deficient mice to a multistage chemical protocol, the animals were resistant to papillomatogenesis [52]. In addition, mating experiments between p53-deficient and transgene mice expressing epidermally targeted v-ras, v-fos, and transforming growth factor α showed an inhibition of spontaneous and TPA-promoted papilloma formation [53–55]. However, transgenic animals expressing a dominant-negative p53 mutation in the epidermis showed an increased sensitivity to two-stage chemical carcinogenesis [56]. While the majority of papillomas in control mice regressed after termination of TPA treatment, the p53-mutated papillomas progressed to carcinomas and metastases. The effect of accelerated tumorigenesis was explained with an increase in genomic instability that resulted from an inhibition of G1 arrest. Taken together, there is little evidence that p53 mutations can act as an initiating event in chemical skin carcinogenesis, as p53 null mice were not prone to spontaneous tumor formation unless they were initiated by treatment with a chemical mutagen. Therefore, loss of p53 function may induce tumor progression rather than initiation.

In contrast to p53, there is strong evidence that activation of the Ha-ras oncogene occurs early in mouse skin carcinogenesis and in fact represents the critical initiation event [57]. Activated Ha-ras has been found in papillomas and carcinomas induced by DMBA, and the activation correlated with a high frequency A–T transversions in codon 61 [58]. Lines of transgenic mice carrying a v-Ha-ras oncogene fused to the regulatory region of the human keratin K1 gene have the properties of genetically initiated skin and develop tumors after promoter application without initiation with a chemical mutagen [53,54]. There is a large body of evidence showing a strong correlation between ras activation and alterations of expression of cell cycle regulating genes, and skin tumor promotion is characterized by selective and sustained hyperplasia leading to specific expansion of initiated cells in papillomas [41].

The observation that tumor-promoting agents like TPA induce inflammation in mouse skin prompted a number of investigations to study the mechanisms of inflammatory mediators in skin tumor development. The eicosanoid pathway, composed of cyclooxygenases and lipoxygenases metabolites, has been identified to be important during tumor promotion. This has been demonstrated in studies using COX and LOX inhibitors [59] and evidenced by several gene-targeting experiments.

The LOX family catalyzes the regio- and stereo-specific insertion of molecular oxygen into polyunsaturated fatty acids by generating 5S-, 8S-, 12S- 12R-, and 15S-hydroperoxyeicosotetrataeconoic acids. Several isoenzymes such as 8S-Lox and 12S-Lox have been found to be preferentially expressed in the epidermis of humans and mice [60,61], and tumor induction studies in mice showed that both the enzymes were overexpressed in papillomas and squamous cell carcinomas [62,63]. The expression correlated with an increased accumulation of the corresponding HETE products. Interestingly, the amount of HETE correlated with the formation of etheno adducts of DNA [64], suggesting that HETE generates an endogenous mutagene that might induce chromosomal damage in neoplastic cells. However, in contrast to 12S-LOX, the epidermis-type (e) 12S-LOX seems to be downregulated during epidermal tumor development indicating an antitumorigenic activity. This could be demonstrated by generating e12S-Lox transgenic animals [65]. Low transgene expression of e12-LOX was correlated with decreased skin tumor response paralleled by an accumulation of the linoleic acid derivate of 13S-HODE, whereas high transgene expression led to an increased skin tumor formation paralleled by the accumulation of the arachidonic acid derivate of 12-S-HETE. These results indicate opposite functions for production of arachidonic acid and linoleic acids in modulation of chemically induced skin carcinogenesis.

In addition, dysregulation of prostaglandin synthesis, which is catalyzed by the cyclooxygenases Cox 1 and Cox 2, is known for a long time to have a critical role in tumor development [66,67]. Using arachidonic acid as substrate, both enzymes catalyze the formation of prostaglandin H, which is converted by prostanoid synthases into biologically active products like PGE2, PGF2, PGI2, and thromboxane A. While Cox 1 is constitutively expressed in all cells, Cox 2 can be induced by various endogenous and exogenous stimuli like growth factors, cytokines, and the tumor-promoting agent (TPA). An overexpression of Cox 2 has been found in various tumor tissues including breast cancer, stomach cancer, colon cancer, and squamous cell carcinoma of skin [68]. Conclusions for the importance of Cox in tumor promotion have been obtained from studies with Cox-deficient animals. The animals exhibited a reduced sensitivity for multistage carcinogenesis. The effect was more prominent in Cox-2-deficient animals than in Cox-1-deficient mice [69]. Moreover, specific Cox 2 inhibitors have been found to suppress both TPA-induced prostaglandin synthesis and tumor promotion [67], and the prostanoid PGF2a has been identified as a tumor promoter in the mouse skin model of multistage carcinogenesis [70].

In conclusion, chemical skin carcinogenesis is the result of a complex interaction of genetic and epigenetic events, and the development of mouse skin models by gene targeting has been and is helpful to identify the critical genes during tumorigenesis. As mentioned in this review, the AhR and its related proteins are important in chemically induced

carcinogenesis. Recently, we have observed that UV radiation can activate the AhR pathway. Therefore, the analysis of AhR function during UV-induced skin carcinogenesis could contribute to a better understanding of the physiological function of the AhR family, in general, and in skin, in particular.

19.8 DRUG ALLERGY

Similar to chemocarcinogenesis, in which small molecular compounds must be bound to the high molecular compound DNA, sensitization requires the presentation of a high molecular weight compound such as a peptide to T lymphocytes, indicating that small molecular weight compounds must be bound to those peptides before they can become antigenic. Drug-induced skin rashes occur in 2–3% of hospitalized patients. The skin is a preferred target organ for B-type reactions. Dangerous reactions are in particular the anaphylactic shock and angioedema as well as bullous drug reactions including toxic epidermal necrolysis (TEN). Most often, the involved drugs are antibiotics—in particular β-lactamantibiotics and sulfonamides—anticonvulsiva, radio contrast media, and cytotoxic agents such as cis-platin derivates. Investigations about the function of T cells in delayed type reactions have improved our understanding of the pathophysiology and led to new diagnostic options and therapeutic concepts. Recently established animal models as well as investigations on the level of antigen-presenting cells may improve the chances of predicting the immunogenicity of small molecular weight compounds.

Drug allergy and ACD are examples of hapten-induced allergic reactions. Because haptens are small molecular weight compounds, this makes it necessary at least in most cases that they be transformed into highly reactive chemical species, which can be bound to peptides. Therefore, it is not surprising that it has become overt that xenobiotica metabolizing enzymes also play a role in the formation of nominative antigens in the hapten-derived allergic reactions. There are several evidences that CYP-mediated metabolites are involved in drug allergic reactions:

- In our own studies, we were able to show that drug-modified microsomes, which contain the main CYP activity, enhance the reactivity of antigen-specific T lymphocytes [71–73]. In addition, Wolkenstein et al. [74] showed that carbamazepine which induced allergic drug reactions quite often—compared with other drugs—is covalently bound with its reactive metabolites to CYP isoforms present in the skin.
- In several cases of drug allergic reactions including cases of toxic epidermal necrolysis to sulfonamides and phenytoin, microsomal antibodies were detected in the serum of the patients, which bind to CYP isoenzymes, which are involved in the metabolism of the drug or microsomal proteins with a position in the microsomal membrane close to the CYP isoenzymes.
- Sensitization to sulfamethoxazole in mice is dependent on the CYP-dependent metabolism of sulfamethoxazole to nitroso-sulfamethoxazole derivatives [75]. This supports the necessity of the processing of the hapten to be bound to peptides and finally to be presented by MHCII receptors to sensitize CD4+ T lymphocytes. In addition, Schnyder et al. [76] proposed an additional model by studying the sensitized T lymphocytes. They found that sulfamethoxazole could directly bind to the HLA–peptide complex stimulating T lymphocytes through an HLA-restricted processing- and metabolism independent pathway. However, under the condition of challenges, MHCI presentations to CD8+ T lymphocytes are also possible and probably circumvent the processes, which lead to a binding with MHCII receptors, which, however, is a prerequisite for sensitization.

19.9 ALLERGIC CONTACT DERMATITIS

Similar to allergic drug reactions, ACD is mediated by small molecular weight compounds. This is a frequently occurring disease and about 6% of the patients who are seen by a dermatologist suffer from it. In addition, this disease has a major socio-medical impact, because it is quite often the reason for an occupational skin disease, which costs, for example, the German health system about 3 billion per year (Diepgen T. L., personal communication).

Allergic contact dermatitis is a form of delayed-type hypersensitivity reaction mediated by T lymphocytes. Up to now, much efforts have been taken in the elucidation of cellular reactions to contact allergens such as nickel and uroshiol to understand the immunological process to define populations at risk and to facilitate hazard evaluations. The ACD as well as other immunological reactions such as drug allergy has two main phases: sensitization and elicitation. During the sensitization phase, the individual acquires a specific immunological memory at the T-cell level to the contact sensitizer after its presentation to them by Langerhans cells in the local lymph node. Upon renewed contact with the sensitizing substance, those sensitized T lymphocytes will be reactivated and this will amount to an inflammatory response, which finally leads to ACD. Multiple cytokines, chemokines, as well as transmembrane signalling proteins are involved in this process and the use of different interactions of potential haptens with these compounds has been tried for hazard evaluations in predicting whether a specific hapten is more or less sensitizing. However, these assays work in some cases but not in others, which makes the predictivity unsafe. One reason may be that these compounds are metabolized and that it is necessary to understand the sensitization process on a more precise molecular level including the enzyme-dependent metabolism of most of these compounds.

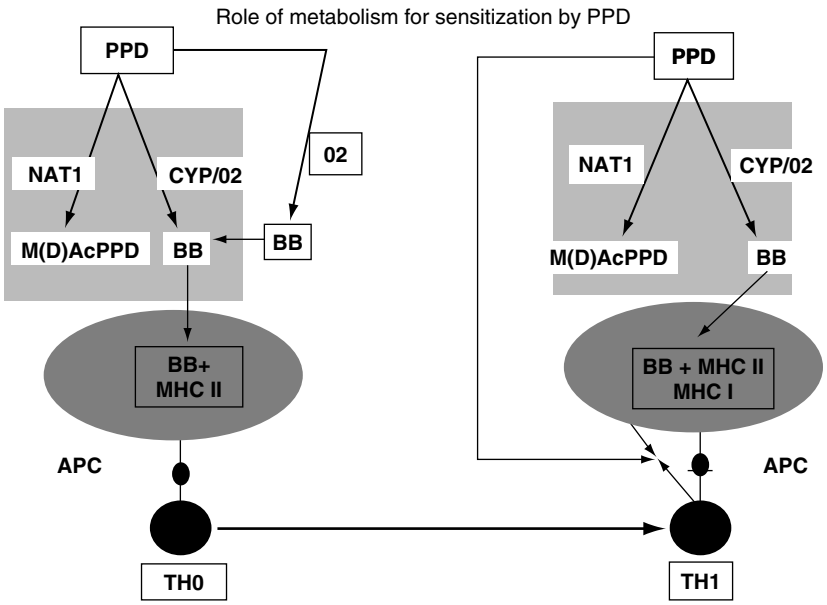


FIGURE 19.2 Metabolic pathways of PPD to BB and its acetylated (Ac) derivatives and its relation to sensitization and challenge by PPD. Reprint of Merk, H.F., Baron, J.M, Heise, R., Fritsche, E., Schroeder, P., Abel, J., Krutmann, J., *Exp. Dermatol*., 15, 692–794, 2006. With permission.

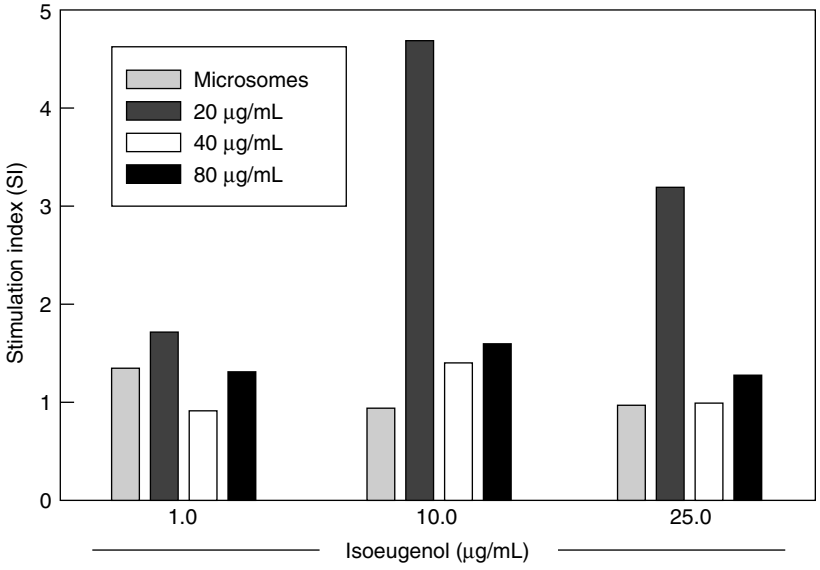


FIGURE 19.3 Effects of fragrance-modified human liver microsomes. To study the involvement of metabolizing enzymes in the presentation of fragrances, antigen-modified human liver microsomes (at indicated final concentrations) were tested for their ability to obtain a polyclonal stimulation of T lymphocytes of a sensitized patient. Proliferation was determined by 3H-thymidine incorporation and is given as the stimulation index (SI). Reprint of Merk, H.F., Baron, J.M., Heise, R., Fritsche, E., Schroeder, P., Abel, J., Krutmann, J., *Exp. Dermatol*., 15, 692–794, 2006. With permission.

19.10 *p*-PHENYLENEDIAMINE

p-Phenylenediamine (PPD) is one of the most frequently encountered contact allergens and up to 50–60% of subjects in certain professions are sensitized. This compound is acetylated in human skin and keratinocytes and alternatively oxidized to Bandrowski's base (BB), which is involved in the allergic reactions to PPD [77,78]. Our own studies demonstrated that PPD itself could be recognized by sensitized T lymphocytes through a processing-independent pathway, whereas its autoxidation product BB required processing and possible metabolism to stimulate T lymphocytes. In addition, the polyclonal response to BB was enhanced by metabolically active enzymes such CYP isozymes [78]. There was no reactivity to acetylated derivatives of PPD, suggesting that this pathway is a detoxifying pathway (Figure 19.2). This acetylation is mediated by the enzyme N-acetyltransferase 1 (NAT1) which is known to be polymorphically expressed in the population leading to high and low capacity to acetylate PPD. Preliminary investigations of the relation of this polymorphism and the likelihood of becoming sensitized to PPD demonstrated at least a trend that slow-acetylators possess an increased risk to develop this contact dermatitis; however, these data are not statistically significant till now.

19.11 FRAGRANCES

Sensitization rates to fragrances are the highest beside nickel sulfate in most countries with western lifestyle [79]. Their ubiquitous distribution leads to unavoidable exposure and

they are present not only in cosmetic products but also in household products, medicaments, food, paper, and paints. There are several examples that fragrances must be oxidized to become the nominative antigen. This may occur by spontaneous oxidation as in the case of D-limonene, abietic acid [80,81], or by enzymatic metabolism, for example, cinnamic alcohol or eugenol [82–85]. In our own studies, we observed that the T-cell stimulation of eugenol- or isoeugenol-sensitized patients was increased in the presence of antigen-modified human liver microsomes (CYP) or recombinant CYP 1A1 in five of seven cases [84] (Figure 19.3). This suggests that metabolically active enzymes such as CYPs may be involved in the activation of fragrances and that the study of those enzymatic pathways may be important to recognize the potential strong antigens by appropriate *in vitro* cell systems or expert systems such as DEREK.

19.12 CONCLUDING REMARKS

In conclusion, the improvement in our knowledge of dermatotoxicologic processes in the skin on a molecular level as well as the possibility to apply new techniques such as the cloning of lesional T lymphocytes [86], transgenic mice [87], and *in vivo* analysis of gene expression in human skin by means of chip technology and quantitative real-time PCR—just to mention some examples—has led to an improved understanding of the toxicological processes. This development allows not only the replacement of older experimental settings but also the use of the easily obtainable skin cells to investigate the principal toxicological processes, which may be valid for other organs as well. An example for this may be the recent suggestion to use real-time (Taqman) PCR to quantify the levels of CYP mRNA in the skin to study phenotypes within the population with differential drug-metabolizing abilities [88]. In this regard, the skin might represent an ideal model organ to assess the interaction of the human body with environmental noxae.

ACKNOWLEDGMENTS

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20 Molecular Basis of Allergic Contact Dermatitis

Jean-Pierre Lepoittevin and Valérie Berl

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20.1 INTRODUCTION

Among the pathological conditions in which chemistry plays an especially important role is contact allergy (Lepoittevin, 1998), and chemical reactions/interactions are involved throughout the biological process that will result in the patient developing delayed hypersensitivity. Thus, the crossing of the cutaneous barrier is mainly controlled by the physicochemical properties of the allergen (molecular volume and lipophilicity). The formation of the hapten–protein complex, which involves the formation of new chemical bonds, is driven by molecular orbital properties. Finally, the recognition between the antigen and the receptors on T lymphocytes can be explained by a discipline undergoing rapid development, that of supramolecular chemistry.

Recently, there has been a major step forward in our understanding of the molecular basis of hapten recognition by T cells. Nevertheless, this does not eliminate the need to understand the characteristics of the preceding processes, as it is true that the properties of a chemical are implicit in its molecular structure.

To cause sensitization, a compound has to penetrate the skin (Roberts and Walter, 1998), where it may be metabolized (Hotchkiss, 1998), and react with Langerhans cell surface proteins to form new chemical structures that are recognized as foreign. We discuss in this chapter the way low molecular weight chemicals can react with skin proteins to form complete antigens, and how these structures could be recognized by T-cell receptors.

20.2 SOME CHEMICAL REMINDERS

Haptens (small molecules with a molecular weight less than 1000 Da) can interact with biological macromolecules by mechanisms leading to the formation of bonds of various strengths (Figure 20.1). These bonds, known as chemical bonds, are the result of electronic interactions between atoms and are characterized by the energy involved. This reflects the bond stability, as this amount of energy must be provided to break the link between the two atoms. In general, a distinction is made between so-called weak interactions, involving energy levels from a few Joule to around 50 kJ/mol, and strong interactions, covalent or coordination bonds, with energies ranging from 200 to 450 kJ/mol.

20.2.1 Weak Interactions

Weak interactions are normally grouped into three main categories: hydrophobic bonds, dipolar bonds, and certain ionic bonds. Although these weak interactions involve modest energy levels and produce structures of low stability, they are nonetheless of great biological importance, as they control virtually all the phenomena of interaction between receptors and substrates.

Hydrophobic bonds represent the ability of organic molecules to organize themselves in water so as to minimize the contact area that they expose to the aqueous solvent. It is, for example, by such means that hydrophobic molecules insert themselves into the phospholipid bilayers of cell membranes

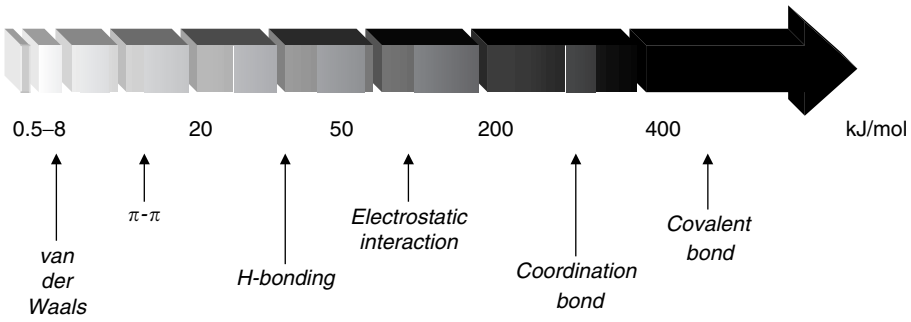


FIGURE 20.1 Energy level of chemical bonds.

and into the hydrophobic regions of proteins or membrane receptors. These hydrophobic bonds, which involve energies in the order of 40–80 J/Å^2/mol, seem, nevertheless, to play an important role in allergies to very lipophilic products (Darley et al., 1977), such as the allergens from poison ivy (*Rhus radicans* L.) or poison oak (*Rhus diversiloba* T.). This could also be of importance for the interactions of haptens with the lipophilic domains of antigen-presenting cells.

Dipolar bonds are electrostatic interactions between permanent or induced dipoles. The electron clouds do not always have a uniform charge density (these variations result from the structure of the molecule), and the zones of high electron density can interact electrostatically with zones of low electron density (permanent dipoles). Electron clouds can also be deformed and polarized as they approach one another, thus creating induced dipoles. These interactions between dipoles, also known as van der Waals bonding, involve energies in the order of 0.5–8 kJ/mol. Hydrogen bond is a special case of dipolar interaction and they occur between a hydrogen atom, bound to an electron-withdrawing atom, and an electron-rich atom. The energy of such bonds can be as high as 25 kJ/mol.

Ionic bonds are based on electrostatic interactions between preexisting and generally localized charges on organic molecules or minerals. Such interactions occur, for example, between the charged amino acids in proteins and are, therefore, important in recognition phenomena.

20.2.2 Strong Interactions

Covalent bonds result when two atoms share a pair of electrons and are classically represented in chemical formulas by dashes. They involve energies in the order of 200–450 kJ/mol and are, therefore, very stable compared with the weak interactions. The two electrons required for bond formation can be contributed by both partners, which is called a radical reaction, or can be provided by one of the atoms, which is especially electron rich, and shared with an electron-poor atom; this case is referred to as a reaction between a *nucleophile* (electron rich) and an *electrophile* (electron poor) center. These two terms, nucleophile and electrophile, represent the capacity of a molecule, or rather an atom of this molecule, to donate or accept electrons to form a covalent bond. Nucleophilic centers are rich in electrons and, therefore, partially negatively charged, while electrophilic centers, deficient in electrons, are partially positively charged.

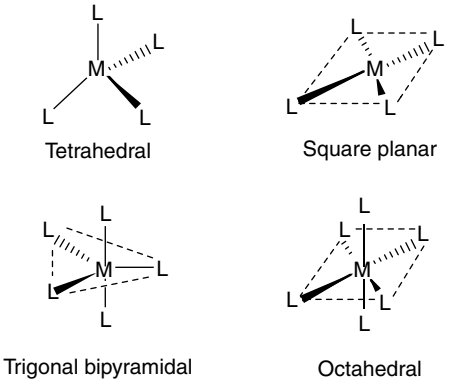


FIGURE 20.2 Examples of geometry for coordinate bonds.

Coordination bonds are another type of relatively strong bond, comparable to covalent bonds; this occurs between metals or metal salts and electron-rich atoms (mainly heteroatoms, such as nitrogen or oxygen). These interactions permit the electron-rich groups or ligands to transfer part of their electron density to the metal and increase its stability. Coordinate bonds are characterized by the number of ligands and by a geometry characteristic both of the metal and of its oxidation degree (Figure 20.2). For example, cobalt (II) (Co^{2+}) is characterized by a tetrahedral arrangement, nickel (II) (Ni^{2+}) by a square planar tetra coordinated arrangement, and chromium (III) (Cr^{3+}) by a six-ligand octahedral arrangement. The number of ligands and the geometry of these coordination complexes determine whether the metals are allergenic and control cross-reactions.

20.2.3 Mechanisms of Bond Formation

The main mechanisms for the formation of covalent bonds involved in contact allergy can be grouped into three main categories: nucleophilic substitutions, on either a saturated or unsaturated center, and nucleophilic additions (Figure 20.3).

Nucleophilic substitution on a saturated center involves the attack by an electron-rich nucleophile on an electron-poor electrophilic center. As the electrophilic carbon already has four single bonds, a new bond can only be formed if one of the existing bonds is broken. The overall effect will, therefore,

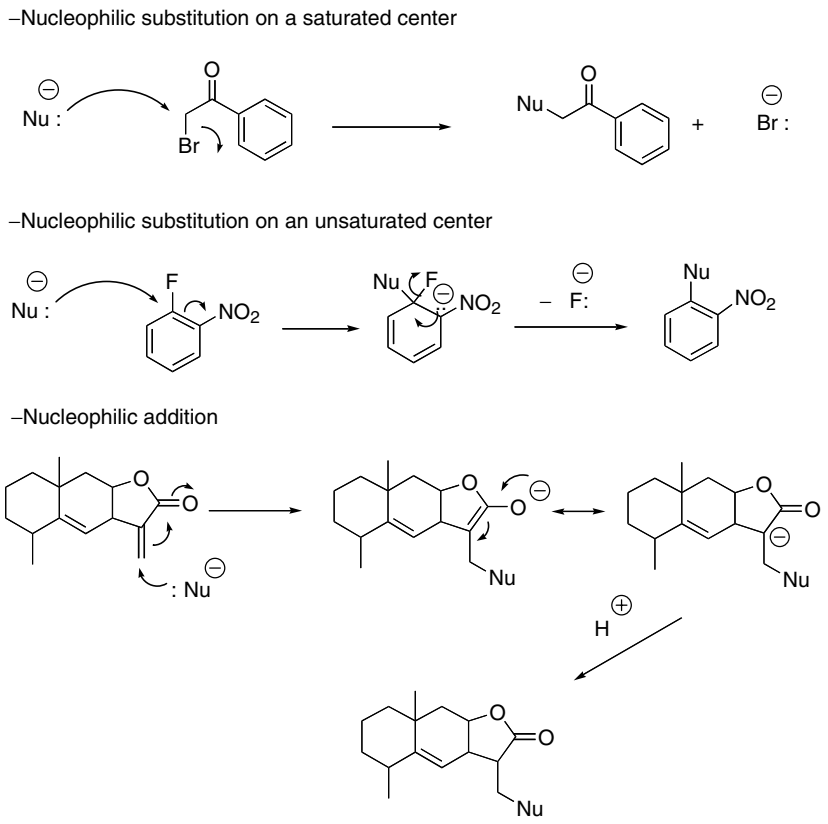


FIGURE 20.3 Principal mechanisms of covalent bond formation in contact allergy.

be a substitution of one of the groups (the leaving group) by the nucleophile.

A *nucleophilic substitution* reaction can also take place at an *unsaturated center* (a carbon with one or more multiple bonds). In this case, although the overall result is again a substitution, the mechanism is slightly different. The presence of a multiple bond allows the formation of a saturated intermediate and the subsequent reformation of the multiple bond permitted by the departure of a leaving group, resulting in the substitution product. This mechanism is illustrated in the aromatic series in which it is all the more favored by attracting groups (e.g., nitro), which stabilize the intermediate.

Nucleophilic addition is simply the addition (with no leaving group) of a nucleophilic atom to an unsaturated electrophilic center (containing one or more multiple bonds). This mechanism is very similar to the first stage of nucleophilic substitution on an unsaturated center, but the absence of a leaving group rules out the reformation of the multiple bond. A saturated compound is thus produced.

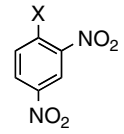
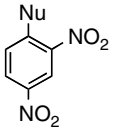
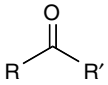
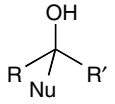
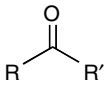
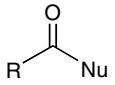
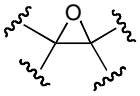
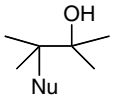
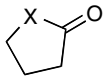
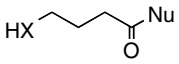
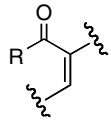
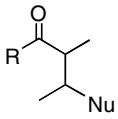
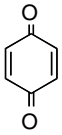
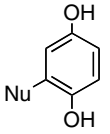
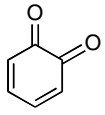
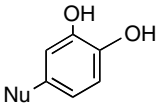
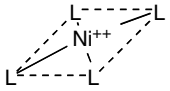
20.3 PRINCIPAL ELECTROPHILIC CHEMICAL GROUPS PRESENT IN CONTACT ALLERGENS

Many chemical groups have electrophilic properties and are thus able to react with various nucleophiles to form covalent bonds. Table 20.1 shows those chemical groups most frequently found in contact allergens and the mechanism by which they react with nucleophilic groups. The previously defined three main types of mechanism, nucleophilic substitution on a saturated center (e.g., alkyl halides and epoxides), nucleophilic substitution on an unsaturated center (aromatic halides or esters), and nucleophilic addition (carbonyl derivatives and α,β-unsaturated systems), can be seen.

20.3.1 Back to Contact Allergy

If we consider biological systems from a chemical viewpoint, it becomes apparent that a very large proportion of structures, especially nucleic acids and proteins, contain many electron-rich groups (those containing nitrogen, phosphorus, oxygen, or sulfur). We can thus consider biological systems as being overall nucleophilic. It is, therefore, not surprising that many biological mechanisms are disturbed on contact with electrophilic chemical substances. Depending on the site of action of these electrophilic molecules, the effect can be mutagenic (Frierson et al., 1985), toxic (Guengerich and Liebler, 1985), or allergenic if the target is the epidermis. In proteins, the side chains of many amino acids contain electron-rich groups capable of reacting with allergens (Figure 20.4). Lysine and cysteine are those most often cited, but other amino acids containing nucleophilic heteroatoms, such as histidine, methionine, and tyrosine, can react with electrophiles (Means and Feeney, 1971). Thus, it has been shown by nuclear magnetic resonance (NMR) studies that nickel sulfate, for example, was interacting with histidine residues of peptides (Romagnoli

TABLE 20.1
Principal Electrophilic Groups Seen in Contact Allergy with Mechanisms of Reaction and the Products

Group	Name	Reaction Mechanism	Product
$R\text{-}CH_2\text{-}X$ X = Cl, Br, I	Alkyl halide	Nucleophilic substitution on a saturated center	$Nu\text{-}CH_2\text{-}R$
 X = F, Cl, Br, I	Aryl halide	Nucleophilic substitution on an unsaturated center	
	Aldehyde; R′ = H Ketone; R′ = alkyl or aryl	Nucleophilic addition	
	Ester; R′ = OR Amide; R′ = NHR	Nucleophilic substitution on an unsaturated center	
	Epoxide	Nucleophilic substitution on a saturated center	
	Lactone; X = O Lactame; X = NH	Nucleophilic substitution on an unsaturated center	
 R = H, R, OR	Aldehyde or ketone α,β-unsaturated	Nucleophilic addition	
	*p*-quinone	Nucleophilic addition	
	*o*-quinone	Nucleophilic addition	
Ni^{++}, Co^{++}, Cr^{IV}	Metal salts	Coordination bonds	

et al., 1991) and that methyl alkanesulfonates, allergenic methylating agents, were mainly reacting with histidine and to a less extent with lysine, methionine, cysteine, and tyrosine (Lepoittevin and Benezra, 1992). If we consider the chemical structure of some allergens (Figure 20.5) in the light of the chemical principles already outlined, it is easy to understand that all of these molecules will be able to react with biological nucleophiles. The so formed extremely stable covalent bonds could then lead to the triggering of delayed hypersensitivity. Again, the previously described three main

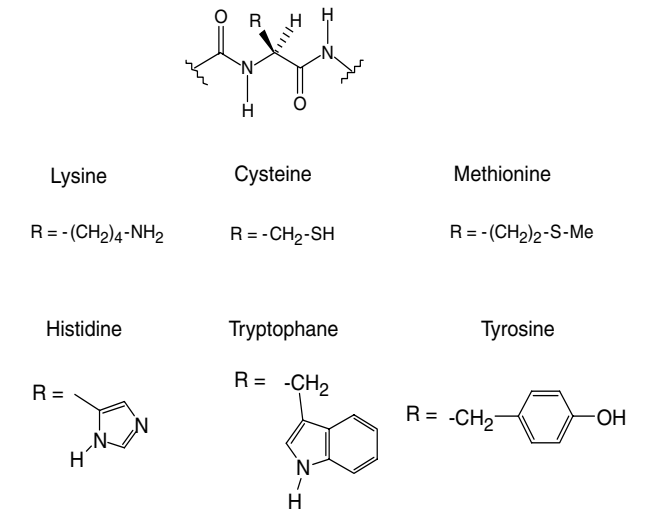


FIGURE 20.4 Principal nucleophilic residues in proteins.

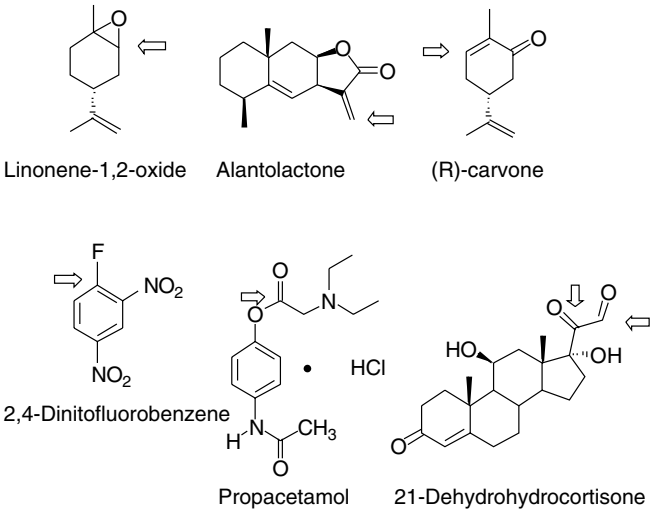


FIGURE 20.5 Examples of sensitizing molecules. The electrophilic center is indicated by an arrow.

types of mechanism for the formation of covalent bonds are seen; the arrows indicate the reactive center of each molecule.

20.3.2 Chemical Selectivity of Haptens for Amino Acids

A direct consequence of the diversity of hapten–protein interactions is the existence of selectivity for amino acid modifications. For example, we have shown that the α-methylene-γ-butyrolactones, the major allergens of plants of the Asteraceae family, principally modify lysine residues (Franot et al., 1993). It has also been shown that not all modifications were antigenic and that the sensitization potential of a molecule is probably more related to its ability to modify some specific residues rather than to modify a large number of amino acids. Thus, the difference in sensitizing potential of two sultone derivatives, an alkenylsultone (a strong sensitizer) and an alkylsultone (a weak sensitizer), which differ only by the presence of a double bond, could be rather explained by the selective modification of lysine residues by the strong sensitizer than by the many tyrosine residues modified by both derivatives (Meschkat, 2001a,b). The same observation can apply to 5-chloro-2-methylisothiazol-3-one (MCI) and 2-methylisothiazol-3-one (MI), the main components of Kathon CG. While both molecules were very reactive toward cysteine residues (Alvarez-Sanchez et al., 2003), the strong sensitizer MCI was also shown to react with lysine and histidine residues in proteins (Alvarez-Sanchez et al., 2004a,b).

These differences, initially purely chemical, seem increasingly to have a major impact on the response of the immune system. The selectivity of the sites of haptenization is directly involved in the selection of the peptide fragments that are presented by the APC to the T cells and thus in the selection of T cell receptors. This selectivity also indirectly controls the level of haptenization of the protein, or proteins. It appears that the epitope density on the surface of the APC directly or indirectly directs the immune response towards Th1 or Th2, high epitope densities directing the response toward Th1 and low densities toward Th2 (Hosken et al., 1995). It is reasonable to ask if this selection of response profile, related to epitope density and thus to hapten reactivity, might not explain, for example, the observed differences between respiratory and skin allergens.

In recent years, the radical mechanism has gained increased interest in the discussion of the mechanism of hapten–protein binding. This mechanism, which has never been firmly established, has been postulated to explain, for example, the allergenic potential of eugenol versus iso-eugenol (Barratt, 1992). More recently, studies indicating that radical reactions are important for haptens containing allylic hydroperoxide groups have been published (Giménez-Arnau, 2002, Bézard et al., 2005).

20.4 METABOLISM, PROHAPTENS, AND PREHAPTENS

Far from being an inert tissue, the skin is the site of many metabolic processes, which can result in structural modification of xenobiotics that penetrate into it. These metabolic processes, primarily intended for the elimination of foreign molecules during detoxification, can, in certain cases, convert harmless molecules into derivatives with electrophilic, and, therefore, allergenic properties. The metabolic processes are mainly based on oxidation reactions via extremely powerful enzymatic hydroxylation systems, such as the cytochrome P-450 enzymes (Mansuy, 1985), but monoamine oxidases, which convert amines to aldehydes, and peroxidases seem to play an important role in the metabolism of haptens. When activated by the production of hydrogen peroxide during the oxidative stress following the introduction of a xenobiotic into the skin, peroxidases convert the electron-rich aromatic derivatives (aminated or hydroxylated) into quinones, which are powerful electrophiles. In this way, it has been proposed that the long-chain catechols, responsible for the severe allergies to poison

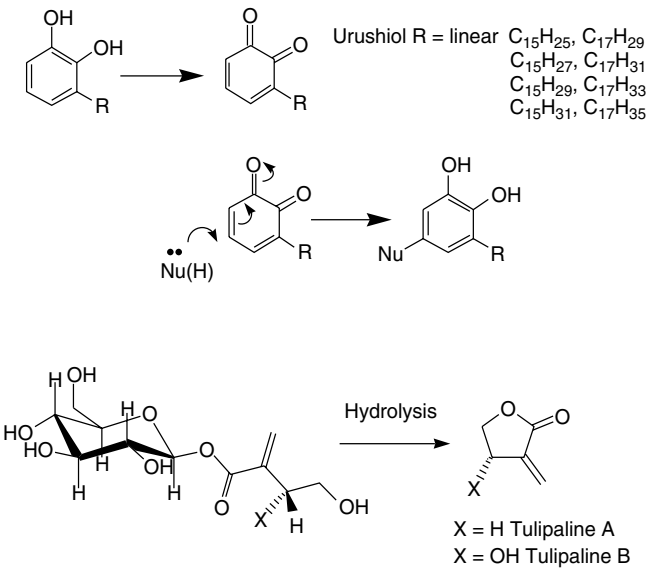


FIGURE 20.6 Examples of prohapten metabolism.

ivy *(Rhus radicans* L.) and poison oak (*Rhus diversiloba* T.), could be oxidized *in vivo* to the highly reactive orthoquinones (Dupuis, 1979) (Figure 20.6). The same applies to paraphenylenediamine or hydroquinone derivatives, such as the allergens from *Phacelia crenulata* Torr. (Reynolds, 1981), which are converted into electrophilic paraquinones. If the formation of such reactive intermediate in the epidermis could explain the sensitizing potential, it is not clearly established if these transformation are linked to specific enzymatic systems or could occur by chemical oxidation with molecular oxygen present in tissues. Metabolic reactions involving enzymatic hydrolyses can also occur in the skin. It is thus that the tuliposides A and B, found in the bulb of the tulip (*Tulipa gesneriana* L.), are hydrolyzed, releasing the actual allergens, tulipalines A and B (Bergmann et al., 1967).

All these molecules, which have themselves no electrophilic properties and cannot, therefore, be haptens but which can be metabolized to haptens, have been referred to as prohaptens (Landsteiner and Jacobs, 1936; Dupuis and Benezra, 1982) and play an important role in contact allergy because of their number and their highly reactive nature. The fact that the structure of the metabolized molecule can be far removed from the structure of the initial molecule can make allergologic investigations even more difficult.

Nonenzymatic processes, such as reaction with atmospheric oxygen, ultraviolet irradiation, or oxygen present in tissues can also induce changes in the chemical structure of molecules. Many terpenes spontaneously autooxidize in air, producing allergizing derivatives. In 1950s it was found that allergenic activity of turpentine was mainly due to hydroperoxides of one of the monoterpene Δ^3-carene (Hellerstrom et al., 1955). This is also the case for abietic acid, the main constituent of colophony, which is converted into the highly reactive substance hydroperoxide (Karlberg, 1988) by contact with air (Figure 20.7). Such an autooxidation mechanism has also been demonstrated for another monoterpene, *d*-limonene,

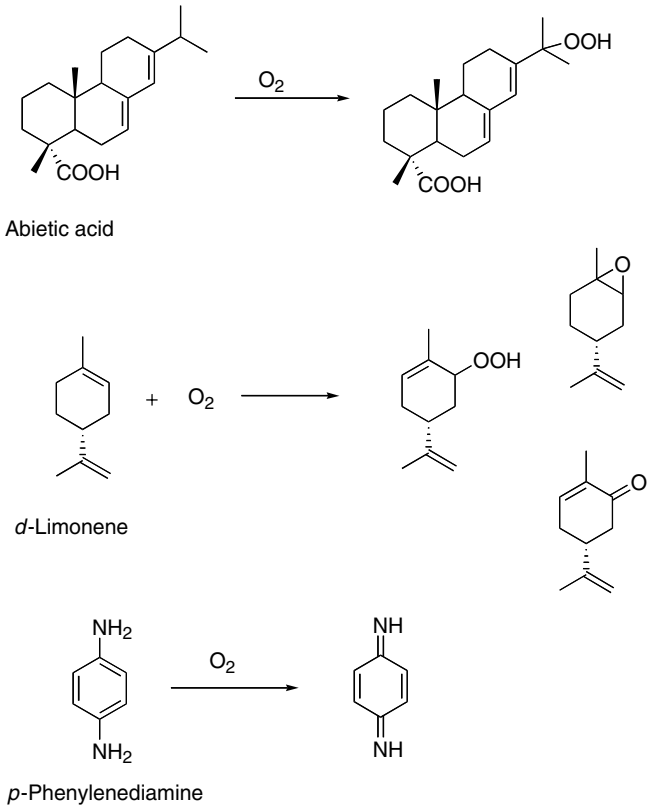


FIGURE 20.7 Examples of prehapten chemical modifications by reaction with air.

found in citrus fruits. *d*-Limonene itself is not allergenic, but at air exposure hydroperoxides, epoxides, and ketones are formed, which are strong allergens (Karlberg et al., 1994).

Since this original classification of sensitizing molecules into "haptens" and "prohaptens," no significant revision has been proposed to take into account more recent developments of the chemical understanding of the sensitization mechanism.

So far these nonreactive molecules have been referred to as prohaptens, even if no specific metabolic enzyme is needed to activate them into haptens. Recent efforts made to develop alternative methods to animal experimentation in the perspective of the European Union ban on *in vivo* testing of cosmetic and toiletry ingredients have shown that this prohapten classification was in fact a break to their development and acceptance.

To make it more clear and stimulate further research on how nonreactive molecules are reacting with proteins to form antigens, a third category named "prehapten" for nonreactive sensitizing molecules transformed into haptens by simple chemical transformation and without requirement of a specific enzymatic system has been proposed (Lepoittevin, 2006). This would also make the development and validation of alternative methods for the identification of sensitizers easier.

20.5 HAPTENS AND CROSS-ALLERGY

The factors that control molecular recognition during the elicitation stage are primarily the nature of the chemical group

and the compatibility of the spatial geometry. Although the first factor (the identity of the chemical group) is very important and serves to define what are commonly called the group allergies, it cannot account for all structure–activity relationships. Receptor molecules are highly sensitive to volume and shape, and molecules must have a similar size and spatial geometry to be recognized by the same receptor. Thus, even though the molecules tulipaline A or B and alantolactone (the allergen of *Inula helenium* L.) bear the same chemical group, α-methylene-γ-butyrolactone, they cannot give rise to cross-allergenic reactions, as their spatial volumes are too different. In contrast, isoalantolactone and alantolactone produce a cross-allergic reaction (Stampf et al., 1982), since they share a homologous chemical group and spatial volume. The term cross-allergy is often misused and should be restricted to the well-defined cases that can be called the true cross-allergies (Baer, 1954; Benezra and Maibach, 1984).

True cross-allergy between a sensitizer A and a triggering agent B can be interpreted in various ways:

- A and B are chemically and structurally similar.
- A is metabolized to a compound that is similar to B.
- B is metabolized to a compound that is similar to A.
- A and B are metabolized to similar compounds.

The identification of cross-allergenic responses can be especially difficult, particularly in humans, in whom the possibility of co or polysensitization should never be ruled out. In addition, the metabolism of molecules can be very complex, and two molecules with a priori little in common can be converted to derivatives that have a similar structure. Thus, derivatives of hydroquinones and *p*-phenylenediamines can be converted into benzoquinone derivatives. It is, therefore, dangerous to draw conclusions from tests without knowing how the substances used are liable to be metabolized. Many reactions described as demonstrating cross-allergy are, without doubt, due to cosensitization (Benezra and Maibach, 1984). Experimental studies in animals are often the only means of being really certain of what happens during recognition. The concept of the prohapten is very important in the interpretation of results in allergy. As the structure of the metabolized molecule can sometimes be very different from that of the initial molecule, it can be difficult to establish similarities of chemical groups and structure.

20.6 APPLICATION TO THE *IN VITRO* PREDICTION OF SKIN SENSITIZERS

In view of the forthcoming European Union ban on *in vivo* testing of cosmetic and toiletry ingredients following the publication of the 7th amendment of the Cosmetic Directive, there is an urgent need to develop *in vitro*/in silico alternative methods. To that respect, our knowledge of the chemistry of skin sensitizers and the way they interact with skin proteins, as the first step of the biological mechanism leading to skin sensitization, could be used as a toxicological end-point. Human serum albumin (HSA) has often been used as a model of choice for protein-binding assay. However, its large molecular weight makes it difficult to use as a screening tool. Therefore, the potential of model peptides to assess the covalent binding of chemicals has been explored. Thus, a GSH binding assay using MALDI-MS to detect the formation of adducts was found to identify 13 of 14 sensitizing chemicals while all nonsensitizers were found unreactive toward GSH (Kato et al., 2003). However, this binding assay based on the detection by mass spectrometry of formed adducts on GSH was not able to detect paraphenylenediamine probably due to the complex chemistry of this molecule. More recently, an approach based on the measure of the depletion of three peptides when reacted with chemicals was proposed (Gerberick, 2004). The proposed peptides were GSH and AcRFAAXAA where X = K or C to cover the cystein and lysine reactivity. Thirty-eight chemicals embracing a wide chemical diversity and classified as extreme, strong, moderate, weak, and nonsensitizers according to the LLNA were tested. The results demonstrate that a significant correlation (Spearman correlation) exists between allergen potency and the depletion of GSH ($p = 0.001$), lysine-peptide ($p = 0.025$), and cystein-peptide (0.020). Thus, by performing a peptide reactivity assay one can reduce the number of compounds that would require testing on an animal model.

20.7 CONCLUSION

The principles that we have just discussed permit a rational approach to the phenomena of contact allergy, but, in actual fact, we often have only indirect evidence suggestive of one mechanism or another available. Although the chemical bases for hapten–protein interactions can be checked in the laboratory by the use of nucleophilic amino acids, small peptides, and model proteins, and although a certain number of steps can be checked, at the present time, no method is available to follow a hapten step by step during the entire immunological process leading to contact allergy. Many points await investigation, but in many cases a "chemical" analysis of the problem does allow us to understand and to foresee cross-allergies and thus to warn the patient about structurally related products.

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21 Photoirritation (Phototoxicity, Phototoxic Dermatitis)

Natalie M. Moulton-Levy and Howard I. Maibach

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21.1 INTRODUCTION

Drug-induced skin photosensitivity is a well-documented phenomenon. Exogenous chemicals and drugs may cause photosensitivity by two main mechanisms: phototoxicity and photoallergy. Both processes occur as a result of an offending exogenous agent combined with light exposure. Multiple chemicals, such as psoralens, fluorescein dye, some thiazide diuretics, and some fluoroquinolones are able to produce both types of cutaneous reactions. It may be difficult to distinguish between these entities, however, they are pathophysiologically distinct processes.

Phototoxicity is much more frequently encountered. It is typically an acute (within minutes to hours), chemically induced nonimmunologic skin irritation requiring light (photoirritation), which is prominent in areas of sun exposure, and clinically resembles an exaggerated sunburn. Edema, pruritis, erythema, increased skin temperature, vesiculation, and desquamation may be present. These signs may followed by long-lasting hyperpigmentation. In the classic form, a large amount of chemical or drug exposure is necessary to induce a phototoxic reaction. Histamine, kinins, and arachidonic acid derivatives such as prostaglandins are released during the inflammatory processes. Histologic changes resemble those that would be seen in sunburned skin with epidermal dyskeratosis and vacuolation, as well as dermal edema and vascular changes. Mononuclear infiltrate may be evident.

Photoallergic reactions are much more rare. In contrast to phototoxic reactions, photoallergies usually appear between 24 and 72 hours after exposure to a small amount of the exogenous chemical. Cutaneous manifestations resemble acute, subacute, or chronic dermatitis with significant pruritis, and affected areas may spread beyond areas of sun exposure. Photoallergy requires previous sensitization to the agent and is believed to be immune mediated. Reactions may result from cross-reaction between related chemicals. After drug cessation, re-exposure to sunlight may cause a reoccurence of the reaction. This phenomenon does not occur with phototoxic agents. Histologic changes include epidermal spongiosis, perivascular lymphoidosis, and mononuclear exocytosis, which may resemble allergic contact dermatitis.

Clinical identification of photosensitivity reactions requires knowledge about skin effects of photosensitizing chemicals and clinical insight gained from practical experience. However, classic morphologic aspects of photosensitivity are not always apparent; prompt and accurate identification of phototoxic and photoallergic dermatoses induced by oral agents may be a challenge to the clinician.

21.2 PHOTOSENSITIZING AGENTS

Naturally occurring plant-derived furocoumarins, including psoralen, 5-methoxypsoralen (bergapten), 8-methoxypsoralen (xanthotoxin), angelicin, and others, constitute an important class of phototoxic chemicals. Bergapten, psoralen, and xanthotoxin are among the more commonly encountered phototoxic agents.

Psoralens are naturally occurring and are synthesized by plants of the Rutaceae (common rue, gas plant, Persian limes, bergamot) and Umbelliferae (fennel, dill, wild carrot, cow parsnip) (Juntilla, 1976). They also occur in a wide variety of other plants, such as parsley, celery, and citrus fruits (Pathak, 1974; Juntilla, 1976). Phototoxicity reactions have been reported to psoralen-containing sweet oranges (Volden et al., 1983) and to common rue (*Ruta graviolens*) (Heskel et al., 1983).

Bergapten is the active component of bergamot oil and is a well-known perfume ingredient whose toxic skin effects have been accorded the name berlock dermatitis. Based on results of

their studies of perfume phototoxicity, Marzulli and Maibach (1970) suggested that perfume should contain no more than 0.3% bergamot, which is equivalent to about 0.001% bergapten, to avoid phototoxicity. Their work also established that bergapten was the only one of five components isolated from oil of bergamot that was responsible for phototoxic effects of the parent material. Limettin (5,7-dimethoxycoumarin), although more intensely fluorescent than bergapten, did not prove phototoxic to human skin. Bergapten phototoxicity continues to occur in some countries where bergapten-free bergamot is not used (Zaynoun et al., 1981), in Norway, from contact with *Heracleum lacinatum* (Kavli et al., 1983), and in Denmark from skin contact with *Heracleum mantegazzianum*, the giant hogweed (Knudsen, 1983).

Xanthotoxin (8-MOP) is effective in treating vitiligo and psoriasis by oral administration or topical application followed by exposure to UVA psoralen plus UVA light (PUVA phototherapy). The *Ammi majus* plant, containing xanthotoxin (8-MOP) in crude form, has been used therapeutically in Egypt since ancient times (El Mofty, 1948). However, at present, PUVA therapy is considered to have carcinogenic potential and warrants caution. Chronic use of this therapeutic regimen enhances prospects of inducing squamous-cell skin cancer, especially in young patients and in those who are genetically predisposed (Stern et al., 1979). This potential has resulted in a reduced use of PUVA phototherapy in the United States (Parrish et al., 1974).

There are a number of agents outside of the furocoumarin family that are phototoxic. Coal-tar derivatives produce occupational contact photodermatitis and phototoxicity in industrial workers and road workers. Anthraquinone-based disperse blue 35 dye caused such effects in dye process workers. Radiation in the visible spectrum activates the dye (Gardiner et al., 1974). Pyrene, anthracene, and fluoranthrene are strongly phototoxic to guinea pigs (Kochevar et al., 1982).

Phenothiazines, such as chlorpromazine, cause phototoxic effects, which have also been ween with oral therapeutic use of amiodarone, a cardiac antiarrhythmic drug (Chalmers et al., 1982). Incidence, time course, and recovery from phototoxic effects of amiodarone in humans were studied by Rappersberger et al. (1989). Antimalarials quinine and quinidine appear to be phototoxic, and some of these have been studied *in vitro* and *in vivo* (Moore and Hemmens, 1982; Epling and Sibley, 1987; Ljunggren and Wirestrand, 1988). Cadmium sulfide, used in tattoos for its yellow color, may be phototoxic (Bjornberg, 1963). Thiazide diuretics were shown to have a phototoxic potential in one study (Diffey and Langtry, 1989), but thiazide-induced phototoxicity is actually rare in clinical practise. There have been recent reports of phototoxicity induced by perforatum hypericum, contained in herbal antidepressant St. John's wort (Schultz et al., 2001). This agent may function through mechanisms including inhibition of proteasome function (Pajonk et al., 2005).

Tetracyclines, particularly demethylchlortetracycline, and also doxycycline, chlortetracycline, and tetracycline, are phototoxic when orally ingested (Verbov, 1973; Frost et al., 1972; Maibach et al., 1967). Doxycycline was reported more potent than demethylchlortetractracycline or limecycline in one human study (Bjellerup and Ljunggren, 1985).

Some fibric acid derivatives, such as fenofibrate, have been reported to exhibit photosensitizing effects *in vivo*. Bezafibrate and gemfibrozil are mildly phototoxic, and clofibrate has shown not to be phototoxic at all (Diemer et al, 1996). Diltiazem has also shown to cause photoxicity in some case reports (Rao et al., 2006).

Fluoroquinolone antibiotics have recently proven to be phototoxic (Ferguson et al., 1993). There have been a number of controlled trials supporting this phenomenon. Fluoroquinolones differ significantly in their extent of phototoxicity. Recently, in a randomized, placebo-controlled study comparing phototoxicity, Dawe et al. (2003) found sitafloxacin to be mildly phototoxic; enoxacin and sparfloxacin proved to be much more photoactive in white subjects. Levofloxacin and placebo failed to show a phototoxic effect. In contrast, among Asian subjects, sitafloxacin failed to demonstrate significant phototoxicity. A randomized-controlled trial supported the fact that lemofloxacin, but not moxifloxacin had phototoxic effects (Man et al., 1999).

It is generally accepted that clinafloxacin $\gg$ lomefloxacin, sparfloxacin $\gg$ trovafloxacin, nalidixic acid, ofloxacin, ciprofloxacin $>$ enoxacin, norfloxacin (Snyder and Cooper, 1999). Perfloxacin and sparfloxacin also appear to result in higher amounts of phototoxicity than ciprofloxacin (Ioulios et al., 2003). It is generally believed that levofloxacin and moxifloxacin are among the least phototoxic drugs in this class.

Antimicrobials, such as sulfonamides, and some fluoroquinolones (enoxacin and lomefloxacin) cause a cutaneous photoallergic reaction, as can sunscreen ingredients, most notably para-aminobenzoic acid (PABA) and its derivatives, and fragrances such as musk ambrette. As previously mentioned, thazides, fluorescein dye, and psoralens are phototoxic, as well as photoallergic.

Multiple case reports suggest that pyridoxine hydrochloride (vitamin B6), may have some photoallergenic activity and have been photopatch tested as positive for this agent (Kawada et al., 2000; Morimoto et al., 1996).

Several psychiatric medications including tricyclics, carbamazepine, and benzodiazepines have shown to be cutaneous photoallergens.

Other miscellanous drugs implicated as photoallergens include amantidine, dapsone, nifedipine, and isotretinoin. However, for a number of these agents, formal data proving their photoallergenic potential are lacking.

21.3 NONSTEROIDAL ANTI-INFLAMMATORY DRUGS

Nonsteroidal anti-inflammatory drugs (NSAID) were the subject of extensive investigations for phototoxic potential following reports that benoxaprofen, a suspended British antirheumatic NSAID, has this capacity (Webster et al., 1983; Allen, 1983; Stern, 1983; Anderson et al., 1987). *In vitro* studies with sheep erythrocytes or human leukocytes suggested a phototoxic potential (Anderson et al., 1987; Pryzbilla et al., 1987).

NSAID that are structurally related to propionic acid have been shown to possess phototoxic potential, whereas certain other types of NSAID, such as tenoxicam and piroxicam were not experimentally phototoxic by *in vivo* or *in vitro* test methods (Anderson et al., 1987; Kaidbey and Mitchell, 1989; Western et al., 1987). The propionic acid-derived NSAID produce unique immediate wheal and are, in contrast, with a much delayed exaggerated sunburn response that typifies psoralen phototoxicity.

Although piroxicam is not phototoxic under experimental conditions involving human test conditions (Kaidbey and Mitchell, 1989), it has been implicated as a possible clinical photoallergic or phototoxic photosensitizer. One explanation for the unexpected photoactivity of piroxicam in skin is that a metabolite of piroxicam is indeed phototoxic when isolated and tested on human mononuclear cells *in vitro* (Western et al., 1987). These positive findings and likely explanation are related to the production of singlet oxygen, as indicated by emission at 1270 nm when the suspect metabolite was irradiated with ultraviolet (UV) *in vitro* (Western et al., 1987; Kochevar, 1989).

Other propionic acid–derived NSAID associated with an immediate phototoxic response are nabumetone, naproxen, and tiaprofenic acid (Kaidbey and Mitchell, 1989; Diffey et al., 1983).

Carprofen (Merot et al., 1983), ketoprofen (Alomar, 1985), benzydamine hydrochloride, topical tiaprofenic acid, suprofen, and possibly piroxicam appear to be photoallergenic. However, further work may be needed to separate, clarify, and identify three possible outcomes—allergy, photoallergy, and phototoxicity—in studies involving NSAID.

The general area of cutaneous reactions to NSAID has been extensively reviewed by Ophaswongse and Maibach (1993).

21.4 MECHANISMS OF PHOTOTOXICITY

Phototoxic and photoallergic chemicals typically exhibit biologic response with the UV area of the electromagnetic spectrum, which is subdivided arbitrarily into UVA (320–400 nm), UVB (290–320 nm), and UVC (200–290 nm). UVA represents the less energetic portion of the spectrum and UVC the more energetic (cytotoxic) area. UVA in the range 320–340 nm (UVA2) is more energetic and more skin damaging than UVA in the range 340–400 nm (UVA 1). *In vivo*, both phototoxicity and photosensitivity are primarily due to UVA range light. However, *in vitro*, phototoxic agents absorb and are activated by both UVA and UVB wavelengths. The cause of this discrepancy is unknown. Some phototoxic chemicals, such as porphyrins and fluorescein dye, absorb visible light (400–800 nm).

Exogenous phototoxic reactions are initiated when a photoactive chemical (one capable of absorbing UV radiation) or one of its metabolites enter viable skin cells. The photoactive chemical may enter into the skin by topical administration or it may reach the skin indirectly by the circulatory system following ingestion or parenteral administration. Some systemically administered and possibly topical chemicals may require metabolic conversion to become photoactive. When the photoactive chemical is in the skin, appropriate wavelengths of light penetrate the skin and subsequently photons are absorbed by, and thereby excite electrons in the phototoxic chemical. This process may lead to formation of unstable singlet or triplet states. As these molecules transfer energy to achieve a more stable state, the transferred energy induces cellular damage and generates inflammatory mediators.

The questions of site and mechanism of action of phototoxic chemicals and the importance of oxygen have been much studied. Some phototoxic agents are oxygen dependant, or photodynamic, whereas others are not. Photodynamic chemicals may transfer their energy to oxygen, exciting it to the singlet or doublet state, thereby exerting phototoxic effects. In its excited state, the photodynamic chemical may react with oxygen and form free radicals. Though mechanisms causing reactions of photoactive drugs are mainly free radical in nature, reactive oxygen species are also involved. Photochemical activity of drugs, such as hydrochlorthiazide, furosemide, chlorpromazine, and some NSAIDS is caused by free radical formation. In other systems, the reactive excited singlet form of oxygen is directly toxic toward lipids and proteins (Moore, 2000).

Studies by Gendimenico and Kochevar (1990) have shown that acridine requires oxygen to produce a lethal (phototoxic) effect on mast cells. (Dermal mast cells are known to participate in cutaneous phototoxic responses initiated by UV and visible radiation.) Chlorpromazine is also thought to be activated by a photodynamic process involving molecular oxygen.

Reactive oxygen intermediates may be a main cause of photosensitivity reactions, which can be stopped by agents that block the production of these intermediate products. Antioxidant supplementation may be beneficial in suppressing phototoxic reactions. Vasoactive amines such antihistamine and serotonin may also play a role in cutaneous phototoxic reactions. Eucosanoids, such as prostaglandins and leukotreines, have also been implicated in the process.

Mathews (1963) showed that toluidine blue requires oxygen to produce its lethal (phototoxic) effect on *Sarcina lutea*; however, oxygen is not needed for the phototoxic effect of 8-MOP on *S. lutea*. In addition, it was found that 8-MOP phototoxicity results in damage to cellular DNA, whereas toluidine kills by action on the cell membrane. Psoralens also do not require molecular oxygen to produce phototoxic effects.

Some photoactive chemicals act on cellular DNA (psoralens, may be tricyclics), whereas others act on cellular membranes (tricyclics). Fluoroquinolones may induce DNA breaks and lead to cell death. Keratinocytes may be the most sensitive cells and melanocytes most resistant (Marrot et al., 2003). The differences in phototoxicity potential may be based on differences in substituent placement on the various chemicals (Hayashi, 2005).

Photoallergic reactions are believed to be cell mediated, with radiation-dependant antigen production, therefore stimulating the immune response. UV energy may cause the drug hapten to find a native protein on epidermal cells, therby forming a complete photoantigen. When the antigen is

formed, the photoallergic process is similar to allergic contact dermatitis, with sensitization of the immune system, and a subsequent cutaneous eruption.

A more complete discussion of mechanisms of photosensitized reactions is given in Spikes (1983).

21.5 ELEMENTS OF THE TEST FOR PHOTOTOXICITY

Tests for phototoxic potential of topically applied chemicals are usually conducted with radiation within the UVA range. Some phototoxic chemicals are activated by wavelengths in the visible spectrum (bikini dermatitis) (Hjorth and Moller, 1976), some by UVB (Jeanmougin et al., 1983), and some (doxycycline) are augmented by UVB (Bjellerup, 1986).

Accurate measurements of radiation intensity and frequency are important prerequisites for work in phototoxicity.

Phototesting procedures include photopatch testing and determination of minimal erythema dose (MED) for UVA and UVB. Photopatch testing may be more useful in detecting photoallergy, and MED may be more useful for testing phototoxic agents. In practice, however, it is recommended to perform both types of testing to ensure comprehensive evaluation.

Among animal models for which photopatch testing has proven useful in predicting human phototoxicity are the mouse, rabbit, swine, guinea pig, squirrel monkey, and hamster, in that approximate order of effectiveness (Marzulli and Mailbach, 1970).

The test material is applied to the skin of a human subject or an animal model (clipped skin of mouse, guinea pig, rabbit, or swine). After a suitable waiting period for skin absorption to take place (several minutes, depending on the rate of skin penetration), the chemical test site is irradiated with UV of appropriate wavelengths. The test site is then examined at 1, 24, 48, and 72 h for evidence of phototoxicity, such as erythema, vesiculation, bullae, and finally hyperpigmentation. A comparison is made between the skin of the test site and control sites (one without chemical and one without light).

Results are modified by factors that affect skin penetration, such as test concentration and vehicle, as well as by duration of exposure and by distance from the irradiation source to the test area.

Some photoirritants (e.g., bergapten) produce clinical phototoxicity when the photoirritant site is irradiated within minutes to 1 h after skin application; with others, irradiation is effective when administered at 24 h.

Phototoxic effects are expected when UV is directed at and absorbed by a phototoxic chemical residing in the skin. This results in a skin reaction with cellular components such as DNA.

One of the earliest indicators of phototoxic potential was based on a paralyzing effect on the cilia of *Paramecium* from acridine plus light, reported by Oscar Raab at the close of the nineteenth century. This test method was later followed by a simpler test involving a lytic effect on red blood cells, as an endpoint for phototoxicity.

The subject of *in vitro* assay for phototoxic effects has recently been reviewed (Rougier et al., 1994).

Recently, reconstructed human epidermis models, such as EpiSkin, ShinEthinc, and EpiDerm, have demonstrated ability to serve as *in vitro* models for phototoxicity testing. Certain models have proven effective in discriminating between phototoxic and nonphototoxic compounds, as compared to *in vivo* data. Several protocols for use are currently available. In the future, data obtained from these models will likely contribute a wealth of information, thereby increasing our knowledge and understanding of photosensitivity.

21.6 HIGHLIGHTS

Investigative studies in photosensitivity require a rudimentary understanding of what constitutes appropriate radiation sources for experimental work, as a first step. Knowledge about safety in the use of radiation equipment is equally important.

Well-calibrated equipment for measuring radiation is another prerequisite, including a recognition that with time and use, equipment changes and requires proper upkeep to ensure its quality in performance.

Filters are sometimes needed to provide an appropriate cutoff of unwanted radiation. Window glass is useful in eliminating wavelengths below 320 nm.

Natural sunlight is filtered by atmospheric oxygen, ozone, clouds, particulates, and other environmental factors including altitude, so that wavelengths below 290 nm are effectively shielded from reaching the earth's surface. Consequently, radiation sources that deliver highly energetic shorter wavelengths in the UVC range are unlikely to be useful in experimental photosensitivity studies involving humans.

The radiation ranges that are of greatest biologic focus in photosensitivity studies are UVA (320–400 nm), UVB (280–320 nm), and UVC (<280 nm). As the Commision de l'Eclairage recommends 315 nm as the cutoff for UVB rather than 320 nm, it is important that the investigative photobiologist identify the system of use. However, a rationale for using 320 nm rather than 315 nm as the cutoff for UVA is given in Peak and van der Leun (1992).

The first rule of photochemistry is that cells are injured or killed when photons of radiant energy are absorbed and energy is transferred to target molecules (Spikes, 1983). Phototoxic effects are therefore produced when absorption wavelengths of the sensitizer are the same as those of the radiant energy source (Grotthus–Draper law).

DNA, RNA, deoxy- or ribodeoxynucleotides, enzymes containing such cofactors, and aromatic and cysteine residues of proteins are typical targets of UV phototoxic damage.

Oxygen may or may not participate in the production of a phototoxic event; however, when oxygen is indeed involved, it is often referred to as a photodynamic action.

Psoralens are among the most frequently encountered phototoxic chemicals, as they are present in many plants. Petroleum products, coal tar, cadmium sulfide, acridines,

TABLE 21.1
Clemicals, Plants, and Drugs with Phototoxic Potential

Topical dyes—anthraquinone, fluorescein dye, disperse blue 35, cosin, methylene blue, rose Bengal, toluidine blue, cadmium sulfide in tattoos
Fragrances—oil of bergamot
Furocoumarins—angelicin, bergapten, psoralen, 8-methoxypsoralen. 4,5, 8-trimethylpsoralen
Plant products—celery, figs, limes, hogweed, parsnips, fennel, dill
Coal-tar components—acridine, anthracene, benzopyrene, creosote, phenanthrene, pitch, pyridine
Systemic antibiotics—griseofulvin, ketoconazole, nalidixic acid, sulfonamides, ceftazidime, tetracyclines, fluoroquinolones
Chemotherapeutics—dacarbazine, 5-fluorouracil, vinblastine, methotrexate
Drugs—amiodarone, chlorpromazine, quinine, quinidine, tolbutamide, diltiazem, fibric acid derivatives, hyerpicum perforatum (St. John's wort)
Diuretics—hydrochlorothizide, bendroflumethiazide, furosemide
Nonsteroidal anti-inflammatories—benoxaprofen, naproxen, piroxicam, tiaprofenic acid, nabumetone
Porphyrins—hematoporphyrin

porphyrins, and other chemicals may also be implicated as causative agents for phototoxic effects. Table 21.1 provides a list of phototoxic chemicals.

Finally, it is suggested that investigators be complete in identifying equipment and methodology that they employ to reduce some of the confusion that may enter and has already entered the literature on this subject.

21.7 CONCLUSIONS

Years of investigative efforts, along with improved methods of measuring and administering radiation, have brought considerable progress in our understanding of various aspects of photosensitivity. We appear to have identified and continue to identify major chemical structures that are currently involved in producing phototoxic and photoallergic effects in humans. We have also gained some insight into some of the mechanisms that are involved. Nevertheless, it is always important to be flexible and aware that time may change some of our present and apparently well-conceived perceptions, as it often does.

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22 Permeability of Skin to Metal Compounds, with Focus on Nickel and Copper

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22.1 INTRODUCTION

Exposure to metals and their derivatives in industrial processing, to their emissions from power plants and from waste deposits constitute a human health risk, both in an occupational setting and in normal everyday activities among the general population. According to EPA's Toxic Release Inventory for the year 2000, metal mining accounted for 47% of their releases into air, water, and land (7.10 billion lb), and of the bioaccumulative releases, mercury and mercury compounds accounted for 36% of the total 12.1 million lb. Besides respiratory and gastrointestinal exposure, traditionally a prime concern in toxicology, also skin contact more recently has gained recognition as an important port of entry for xenobiotics into the living organism. Cases of morbidity and mortality due to exposure of the skin to toxic agents has contributed to such a shift in attention (Plueckhahn et al., 1978; Toribara et al., 1997).

The skin is permeable to a wide range of chemicals; it can function as a barrier, a filter, or as a potential reservoir for their accumulation and source for their slow release. Over the past years, skin diffusivity of organic chemical structures has proven amenable to mathematical modeling, and their bioavailability has become predictable with increasing accuracy using mathematical models to describe quantitative structure–permeability relationships (Flynn, 1990; Potts and Guy, 1995). Metal compounds, however, defy such modeling and predictability; this is especially true for strongly electropositive metals as they avidly react with electronegative constituents they encounter along the path of diffusion through the skin. A number of confounding factors come into play expressing their idiosyncrasy, which impact both rate and route of skin penetration, the importance of which is not estimable with our present knowledge. What complicates the picture further is the interdependence among these multiple factors. Those which have an obvious bearing on the process of metal diffusion and which have to be taken into account if one were to anticipate degree of their penetration through biological membranes for purposes of dermal exposure assessment are briefly discussed in Section 22.3. Those perceived as critical for skin diffusion are discussed under two headings: those proper to the metals themselves, the exogenous factors, and those inherent in the barrier tissue as part of the target organism itself, the endogenous factors. These factors are illustrated with observations for the metals nickel and copper in particular. In the following, the term metal applies equally to the elemental state, as well as the ionized form.

22.2 DESCRIPTORS OF SKIN PERMEABILITY

22.2.1 The Permeation Coefficient Kp and Fick's First Law of Membrane Diffusion

The most commonly used descriptor to characterize the diffusion of chemical compounds is the permeation coefficient Kp. It is determined experimentally by measuring the rate of diffusion through excised skin in a one-chamber diffusion cell (Franz, 1975) or the flow-through two-chamber cell (Bronaugh et al., 1986); the latter allowing automatic sampling of the receptor phase. The *in vitro* technique is used for determining quantitative percutaneous absorption data of potentially toxic metal compounds through human skin in particular. The solute passes from the fixed higher concentration medium in the donor chamber by passive diffusion into the less concentrated solution in the receptor chamber. The advantage of such diffusion experiments lies in the standardized expression of the quantitative results when Fick's first law of diffusion is applicable (Fick, 1855). Formulated to characterize passive diffusion of compounds across membranes, in general, Fick's law has been shown to also apply to passive diffusion of xenobiotics through the SC of the skin, and with restrictions also applicable to the skin's permeability for metal compounds. That law states that, if the permeation process from an "infinite" reservoir reaches the point of steady state equilibrium, that is, the concentrations in the donor and receptor phases remain constant over time, the measured steady-state flux Jss describes the amount of permeant per unit time and area, usually expressed as $mg/h/cm^2$. The permeability coefficient or permeation constant Kp, typically in cm/h, is then calculated from the Jss value divided by ΔC, the concentration gradient between donor and acceptor phase. In a diffusion experiment conducted under dynamic conditions, that is, where the permeant reaching the receptor fluid is constantly removed, C in the receptor compartment is zero, and the resulting calculation then becomes $Kp = Jss/C$.

The technique is easily standardized and allows the determination of Kp through skin or other membranes as long as the barrier properties are not affected by either permeant or carrier solvent. This implies that the permeant, in this case the metallic ion, may not react with barrier material, thereby changing barrier permeability. Because heavy metals are electrophilic and avidly bind to electron-rich moieties present in epidermal tissue, such as oxygen, nitrogen, or sulfur, this prejudices the general validity of Kp values (discussed later). Nevertheless, flux values for metal compounds *in vitro*, if obtained under conditions of steady state from an infinite reservoir and normalized for concentration, are the most reliable descriptor available for the skin penetration of such metals also, and the calculated Kp serves as a convenient parameter for putting their diffusion in context with that of other more conforming permeants.

22.2.2 Percentage of Dose Absorbed

The optimal *in vivo* method for the determination of skin absorption is the one developed by Feldmann and Maibach (1969). It yields large part of the data so far available on skin penetration by drugs and pesticides, using human volunteers or monkeys.

In human studies, following topical application of the permeant, the plasma levels of test compounds are low and the use of radiolabelled compounds becomes necessary.

The compound labeled with carbon-14 or tritium, or a metal isotope, is applied to the skin in a minimal volume of a volatile solvent that is left to evaporate, and the total amount of radioactivity excreted is then determined. The amount retained in the body is corrected for by determining the amount of radioactivity excreted following parenteral administration of the compound. The resulting radioactivity value is then expressed as the percent of the applied dose absorbed. Fick's postulates for membrane diffusion are not met there. A concentration of the permeant cannot be defined and neither is a steady state equilibrium observable with this method; the permeability coefficient thus cannot be calculated. Nevertheless, for purposes of risk assessment the indication of potential (whole-body) absorption is an important factor as it gives a measure for human (worst case) exposure.

22.2.3 Predicting Absorption through Stratum Corneum Analysis

The predictive method through which the systemic uptake of a permeant can be derived from the amount that diffuses into the stratum corneum (SC) after a limited time of exposure *in vivo*, developed by Rougier (Rougier and Lotte, 1987a), is only applicable for compounds with Fickian behavior. It cannot be used for electrophilic metals, which react with barrier tissue that, by altering its barrier properties, renders in-depth diffusion unpredictable. In addition, certain metals such as copper or zinc are essential trace metals subject to homeostatic controls, which appear to determine deposition or mobilization in function of fluctuating body burdens.

SC stripping and analysis is used for other purposes, however, and can be applied in the investigation of metal diffusion into the SC and beyond (Hostynek et al., 2001a,b). Tracing the concentration profiles of permeants in SC has been rendered facile by using that semiinvasive method and by state-of-the-art analysis with inductively coupled plasma atomic emission spectroscopy (ICP-AES) and inductively coupled plasma mass spectroscopy (ICP-MS). With this method it is now possible to analyze for the presence of elements in trace amounts in skin tissues and other biological materials to a level of 0.5 ppb (0.5 µg/kg), making the use of radioisotopes unnecessary. The method makes it possible to estimate actual metal concentration profiles to the level of living epidermal tissue.

22.3 PRINCIPAL FACTORS DETERMINING SKIN PERMEATION BY METAL COMPOUNDS

22.3.1 Exogenous Factors

22.3.1.1 Dose

Review on the diffusivity of transition metals shows that their diffusion is not necessarily dose-related; this appears to be one of the several effects attributable to the electrophilic properties, which causes them to form stable bonds with proteins of the skin. In some cases, absorption at first increases with increasing dose, then reaches a plateau value and decreases again with a further increase in concentration, as was recorded for the diffusion of sodium chromate through guinea pig skin *in vivo* (Wahlberg, 1965). For others, such as potassium chromate and human epidermis *in vitro*, absorption steadily decreased with increasing dose (Fitzgerald and Brooks, 1979).

22.3.1.2 Vehicle

A factor for consideration when choosing a vehicle for percutaneous penetration studies is the effect the solvent will have on the skin membrane and thus its barrier properties. The choices may be restricted for the sake of adequate solubility of the permeant. Petrolatum, for instance, is a poor solvent for metal salts, where the permeant remains suspended in fine particles affording less than ideal uniformity in skin contact; however, petrolatum has an occlusive effect, which increases skin hydration and thus promotes diffusion of hydrophilic compounds. Another solvent that enhances penetration is dimethylsulfoxide. It appears to modify intercellular solute diffusion to include the transcellular path (Sharata and Burnette, 1988).

22.3.1.3 Counter Ion

Intuitively, size of permeant is critical for its rate of diffusion through biological membranes, aside from other factors that may play a part in the process. A measure of the effect of the counter ion is the degree of irritancy (and thereby diffusivity) certain metals salts have on contact with the skin, investigated with nickel and chromium with the purpose of optimizing skin patch-test materials for immunological diagnostic testing (Shabalina and Spiridonova, 1988; Wahlberg, 1990, 1996; Lansdown, 1991).

Depth–concentration profiles of a number of different nickel salts in the SC demonstrated the difference in their diffusivity in function of the counter ion. The profile was made visible by the standard protocol of sequential tape stripping of the SC following application of the chloride, sulfate, nitrate, and acetate on the skin of human volunteers (Hostynek et al., 2001b). Following single open application at 1% nickel concentration, the skin was tape stripped 20 times and the strips analyzed for metal content by ICP-MS. The concentration gradient between the superficial and deeper layers of the SC varied distinctly from one salt to the other: acetate $>$ nitrate $>$ sulfate $>$ chloride. The concentration-versus-depth profiles obtained using this method confirmed that the counter ion in nickel salts plays a role in its passive diffusion through the SC, suggestive of ion pairing. The steep initial concentration gradient observed for all four nickel salts also demonstrated the differential formation of a reservoir in the outermost layers of the SC.

22.3.1.4 Molecular Volume

Size of a permeant plays a decisive role in its rate of diffusion through a biological membrane, regardless of other factors

that may be part of the process. Diffusion of a cation is necessarily tied to the diffusion of its counter ion, that is, diffusion of a metal will also proceed as an ion pair, otherwise an electrical potential builds up, which will inhibit further diffusion. In a number of experiments with four nickel salts, the nitrate as the more lipophilic one was the only salt that penetrated the SC at a slow but steady level, leading to the hypothesis that more lipophilic compounds choose the intercellular lipid bilayer as diffusion pathway. For confirmation, diffusion of nickel chloride was compared with that of the di-octanoate as a limit case of lipophilicity. Results, however, showed that rather than polarity, molecular volume is the decisive factor for membrane diffusion. A statistically significant difference appeared between the permeation of the chloride compared to the octanoate ($P = 0.044$). The slower diffusion of the octanoate is likely due to the molecular volume increment of the two octanoate groups, an effect which in this case overrides the effect of polarity (Hostynek, 2003).

22.3.1.5 Nature of Chemical Bond and Polarity

To the degree to which metals form compounds ranging from inorganic (ionic) to organic ligands, bonds increasingly assume covalent character, and their penetration characteristics become similar to those of common organic nonelectrolytes. The lipophilic category, mainly alkyl and aryl derivatives of the more toxic metals, thus represents a major risk in chemicals manufacture due to their ease of skin penetration. The polarity of nickel salts, as measured by their solubility in the nonpolar solvent *n*-octanol at 22°C, increases in highly significant intervals ($p < 0.0005$) in the sequence: nitrate < chloride < acetate < sulfate, ranging over four orders of magnitude (Hostynek et al., 2001b). The effect of counter ion and polarity on the diffusivity of those nickel salts became evident from the slope of the depth–concentration profile obtained through SC stripping and from the area-under-the-curve (AUC) values for the four salts, a measure for the amount retained in the SC: for the nitrate, the AUC was significantly higher (AUC = 26.1 ± 1.2; $p < 0.0005$) than were the totals for the other salts applied at comparable Ni (II) concentrations (AUC = 3.3 ± 0.6 to 18.5 ± 4.1). A reasonable explanation would be the choice of an alternate diffusion pathway and deposition by the more lipophilic nitrate, that is, via the intercellular lipid domains. The salt with the most covalency may thus achieve greatest diffusivity through the SC barrier over time.

22.3.1.6 Valence

The outer electron shell of elements expresses their valence, determines their size and electropositivity. As a consequence, these two associated factors effectively determine diffusivity: degree of steric hindrance retarding penetration, and bond formation with electronegative molecular functions in proteins, which result in deposits.

In transition metals such as copper, the five d electron orbitals, each able of accepting a pair of electrons, are being filled with electrons while an outer s electron shell of slightly lower energy is most often complete with two electrons.

Copper has a single s electron outside the filled 3d shell. Removal of this electron gives rise to the Cu(I) oxidation state with d^{10} electron configuration, while removal of another electron yields the more common Cu(II) oxidation state with the $3d^9$ electron configuration. Although these oxidation states can interchange easily as the equilibrium 2Cu(I) g Cu(0) + Cu(II) can be readily displaced in either direction; further oxidation to Cu(III) is difficult.

22.3.1.7 Depot Formation

Nickel and copper ions present examples of highly electrophilic ions, which readily complex with SC proteins, leading to depot formation. *In vitro* diffusion experiments with several copper complexes showed that a substantial portion of the permeant is retained in the SC, epidermis and dermatomed skin, membranes tested for that purpose (Hostynek, unpublished data). Such retention in the SC is a fair indication of the exogenous agent (copper), which under homeostatic control will eventually become systemically available. For that reason also, measuring diffusion rates only presents part of the overall picture, as the chemical absorbed into the SC will continue to diffuse into the viable tissue, even after exposure has stopped. In that sense, the SC is more than an accumulation of dead keratin, as it fulfills the multiple functions of barrier, reservoir, and filter, depending on the physicochemical nature of the permeant. The amount of material accumulated which, in most cases, can become available for absorption, is appropriately referred to as the SC reservoir (Schaefer and Redelmeier, 1996).

22.3.2 Endogenous Factors

22.3.2.1 Age of Skin

The permeability of the skin for xenobiotics appears to change with advancing age, according to most observations in a decreasing mode. This is attributed to a diminishing blood supply and also to decreasing lipid content of aging skin (Roskos et al., 1989). Investigation of skin permeability to a nickel salt versus a nickel soap using dermatomed skin under identical experimental conditions, including anatomical site, confirmed the decreasing trend in skin diffusivity with age, specifically between skin from a young (age 16) and an older source (age 64). The rate of nickel diffusion for both types of compounds was over two orders of magnitude slower in the older skin: $Kp = 9.8 \pm 4.9 \times 10^{-3}$ versus $6.2 \pm 4.3 \times 10^{-5}$ for $NiCl_2$, and $1.4 \pm 0.57 \times 10^{-3}$ versus $0.9 \times 10^{-5} \pm 0.07$ for the nickel dioctanoate. Remarkably, the ratio between the "young" and "old" values remained constant at a value of 7 for both compounds (Hostynek, 2003).

22.3.2.2 Anatomical Site

Since penetration of electrolytes appears to occur mainly through the skin's appendages, diffusion in hairy areas may be at an advantage, although absorption was also observed through the palm of the hands devoid of hair follicles; route of diffusion there were probably the sweat ducts (Feldmann, 1967).

The effect of anatomical site on the *in vivo* absorption into the SC was studied using tape stripping. Following single open application of nickel chloride on the arm and back over 24 h application sites were stripped 20 times and the strips analyzed for metal content by ICP-MS. At the identical concentration of 314 μg/cm^2, the two sites showed steep, although differing depth–concentration gradients, declining toward deeper SC layers with significantly different areas under the curve : 30.8 for skin on the back versus 62.4 on the arm ($p < 0.0005$), an indication of heightened diffusivity (Hostynek et al., 2001b).

Anatomical differences in penetration of xenobiotics have been observed by several authors. A ranking could be established for different sites, in decreasing order: scrotum-forehead-postauricular-abdomen-forearm-leg-back (Wester and Maibach, 1980; Rougier et al., 1986; Wahlberg, 1996). Such differences were attributed to regional SC thickness and shunt density, but also to intercellular lipid abundance and composition, which play a pivotal role in diffusion (Loth et al., 2000; Elias, 1983; Lampe et al., 1983; Rougier et al., 1987; Guy and Maibach, 1984). Site dependence becomes apparent in effects that depend on diffusivity, such as inflammatory reactions (Wahlberg, 1996), or elicitation of contact allergy, the latter particularly leading to false-negative reactions when hypersensitive subjects are patch tested on the less penetrable skin on the back rather than on the antecubital fossa on the arm (Basketter and Allenby, 1990; van Strien and Korstanje, 1994; Seidenari et al., 1996a,b; Simonetti et al., 1998).

22.3.2.3 Homeostatic Controls

Essential elements such as copper present in the SC, epidermis, and dermis are kept in equilibrium by homeostatic control mechanisms, which play a part of overall physiological dynamics of micronutrients and also appear to regulate the body burden of xenobiotics. These mechanisms ascertain maintenance of equilibria necessary for optimal functioning of the organism, for example, by preventing undue loss due to perspiration through reabsorption as occurs with sodium in particular (Cage and Dobson, 1965). They can also act as surveillance capable of sequestering toxic levels of certain metals, for example, complexing copper with metal-binding proteins such as metallothioneins and coeruloplasmin. Particularly *in vivo* dermal absorption experiments thus should account for such natural processes, which may counteract passive diffusion, attaching a degree of uncertainty on the permeability constants measured.

22.3.2.4 Skin Tissue Section

Comparison of Kps determined through different skin strata *in vitro* by different authors shows that penetration by nickel chloride is slowest through the SC (Samitz and Katz, 1976; Bennett, 1984; Fullerton et al., 1988a; Emilson et al., 1993); from the Kp of 10^{-7} cm/h in the SC (Tanojo et al., 2001), it progressively increases toward full thickness skin, with a maximum seen in dermatomed skin, Kp = 10^{-4} cm/h (Hostynek, 2003), a rate which is three orders of magnitude higher than that in the SC.

22.3.2.5 Role of Skin Shunts

A number of direct or indirect observations speak to the role of shunts in electrolyte diffusion through skin. Penetration through sweat ducts can occur within 1–5 min following exposure, with no comparable transport occurring via the transcellular path in that time span (Abramson and Gorin, 1940; MacKee et al., 1945; Shelley and Melton, 1949). Poral transit by nickel salts in particular can become manifest in clinically follicular inflammation or punctate erythema on patch testing (Menné and Calvin, 1993; Kanerva and Estlander, 1995).

An argument that also speaks for a relatively rapid penetration of nickel via shunts is the finding by Fullerton that when all strata of full-thickness skin had been analyzed by stripping following an *in vitro* diffusion experiment, only 64% of the dose were accounted for (Fullerton, 1988b). The unaccounted-for portion may be explained by shunt diffusion, which eludes strip analysis. At higher doses of nickel nitrate applied on the ventral forearm *in vivo*, strip analysis of the SC also accounted for only 75% of the dose (Hostynek et al., 2001b).

22.3.2.6 Metabolism in the Skin

Metabolic activity, including redox reactions by metals in the organism's tissues, such as the skin, can change the valence *in situ* and significantly alter the diffusivity of ions. Both Cr(III) and Cr(VI) penetrate the skin, with hexavalent chromium (as CrO_4^{2-}) usually the better penetrant; Cr(VI), present as chromate $(CrO_4)^{2-}$ and dichromate $(Cr_2O7)^{2-}$, the most injurious to human health and a carcinogen, applied to the skin is converted into the more electrophilic Cr(III) during transit (Gammelgaard et al., 1992). While chromate and dichromate ions (CrVI) do not complex with organic substances, Cr(III) is inhibited in its diffusivity due to formation of stable complexes with epithelial and dermal tissues (Samitz et al., 1969). The skin diffusion constant Kp, through human skin *in vitro*, for Cr(VI) falls into the 10^{-3} cm/h range, while that for Cr(III) is of the order of 10^{-5} cm/h (Fitzgerald and Brooks, 1979).

22.4 METHODOLOGY

22.4.1 Analytical Techniques

22.4.1.1 Inductively Coupled Plasma–Atomic Emission Spectroscopy

ICP-AES permits detection of metals at the trace amount level, which obviates the use of radioisotopes (Di Pietro, 1988). For the detection of nickel or copper, the current quantitation limit falls in the 5–10 ppb (μg/L) range, a factor of 5 above the true instrumental detection limit as defined by the United States Environmental Protection Agency (US EPA). In practice, ICP-AES is used for analysis of elemental levels at and above the 1 ppm levels, concentrations where an ICP-MS instrument would be swamped, leading to experimental problems.

Quantitative detection of metals with this method is accomplished by ionization of elements in an atmosphere of inductively coupled argon plasma, maintained by the interaction of a radio frequency field and ionized argon gas. In a sample aerosol (e.g., a vaporized metal salt solution), atoms and ions are activated at 6000°C to an unstable energy state, and as they revert to their ground state again, they emit light of characteristic wavelength and intensity, which can be measured.

22.4.1.2 Inductively Coupled Plasma–Mass Spectrometry

Complementary to ICP-AES, ICP-MS is a technique applicable to μg/L (ppb) concentrations of many elements in aqueous medium upon appropriate sample preparation of biological materials. Reliability of the method for elemental analysis is based upon multilaboratory performance tests when compared to results obtained with either furnace atomic absorption spectroscopy or ICP-AES.

ICP-MS measures characteristic emission spectra of ions produced by a radio frequency inductively coupled plasma using optical spectrometry. Compound to be analyzed, present in liquid form, is nebulized and the resulting aerosol transported by argon gas into the plasma torch, also at 6000ºC. The ions produced are entrained in the plasma gas and introduced, by means of a water-cooled interface, into a quadrupole mass spectrometer. The ions produced in the plasma are sorted according to their mass-to-charge ratios and quantified with a channel electron multiplier.

Determining the permeation constants of nickel and copper compounds in solution is possible by this method at the sub-ppb level, obtained upon exposure of human skin *in vitro* in the collected receptor fluid (Tanojo et al., 2001), or from *in vivo* experiments upon digestion of the SC tape strips (Hostynek et al., 2001b).

In *in vivo* experiments, sequential adhesive tape stripping has been used to characterize the penetration of nickel and copper in the SC of human volunteers, applied as the salts or as the metal itself. Exposure areas are stripped to the level of the glistening layer at given intervals postdosing, and the strips analyzed for metal content by ICP-AES or ICP-MS. The metal concentration/depth profiles obtained give clear indication of diffusion depending on time of exposure, and on counter ion of the salt when appropriate, anatomical site, and on the concentration applied (Hostynek et al., 2001a,b).

22.4.2 Diffusion Experimental

22.4.2.1 *In Vitro* Diffusion of Salts

Glass low-volume flow cells exposing a skin application area of 0.8 cm^2 are used (Reifenrath et al., 1984). Cells are mounted in the wells of aluminum cell holders (Stratacor, Richmond, California), whose internal channels are perfused with water at 37°C from a circulating water bath. The cell holders are mounted on a fraction collector (Retriever IV, Isco, Lincoln, Nebraska). The collector's movement is controlled by an external programmable timer (Stratacor), so that receptor fluid exiting the flow cells is fractioned into 12.4 h collection intervals. Distilled water containing 1 mL/L of gentamycin sulfate solution (l00 mg/mL, Sigma, St. Louis, Missouri) is pumped through the cells at approximately 2 mL/h with a 12-channel peristaltic pump (Van Kel Industries, Edision, New Jersey).

Frozen split-thickness human skin is thawed, cut into circles with a #15 cork borer, mounted on the diffusion cells and the donor chambers clamped in place. Eight cells were typically set up at one time, with four cells used for each of the two treatments.

Test solutions (l.0 mL) are placed in the donor chambers, which are then closed with ground glass stoppers and sealed with Parafilm to prevent evaporation of donor solution.

Receptor fluid samples are collected in 20 mL polyethylene screw-cap scintillation (LSC) vials (Maxi-vial, Packard Instruments, Downers Grove, Illinois). Samples are stored at 4°C until analysis. At completion of an experiment, the donor cell contents are pipetted into LSC vials. The donor chamber and skin surface are rinsed (3 × 2 mL) with distilled water and the rinses saved LSC vials. Skin samples are removed from the cells and placed in 20 mL glass LSC vials. Nitric acid (0.75 g) is added to each skin sample and the vials heated (50–60ºC) to effect dissolution. After cooling, samples are diluted with 15 mL distilled water to give a final nitric acid concentration of 3.5% required for ICP analysis. Samples are filtered prior to analysis (Tanojo, 2001).

22.4.2.2 *In Vivo* Diffusion of Salts

Nonatopic in good health and no history of allergies or significant skin disease are selected for the study. As a rule, replicate experiments for statistical purpose are conducted on the same volunteer to minimize experimental variability. Also for each of the parameters investigated, the stripping experiment is conducted on the same volunteer. Salts are dissolved in methanol, targeting a metal content of 0.001–1%. The actual metal concentration in the test solutions is determined by ICP-AES analysis, prior to application, to allow accurate interpretation of results. Methanol is chosen as a model vehicle with the intent to minimize disruption of SC membrane integrity, to optimize volatility and thus expedite open application, and for adequate solubility of nickel salts.

Prior to application of test solution, the targeted area of the skin is cleansed by threefold wiping using cotton swabs moistened with deionized water, then dried with a stream of compressed air for 30 s. 100 mL of the solutions were applied on a 2.83 cm^2 area of the skin using a shallow glass cylinder (1.9 cm inner diameter) for containment, to the base of which a film of silicone grease has been applied to prevent leakage. Following application of the test solution, a gentle air stream (compressed air) was directed at the application site through a plastic tube to enhance solvent evaporation, usually complete within 60 s. During the experiment, care is taken not to touch the area of application; or exposure times of 3 h and longer the application sites were covered with a rigid, perforated plastic shield open at both ends to ensure free air circulation but at the same time preventing mechanical abrasion. The shield is

held in place by taping it to the skin, the tape strips being applied transversally across the shield in such a way as to not cover the air vents. At the end of the dosing period, in several experiments, the application sites are wiped clean with cotton swabs to remove residual test material remaining on the surface prior to tape stripping. Repeated wiping with a water-moistened cotton swab is followed by a dry swab, and finally an air stream is passed over the skin surface for 30 s. The combined swabs are placed in a glass vial for separate extraction and nickel analysis.

Adhesive tape of 2.54 cm width is cut in 5 cm strips, and the SC is stripped according to the following protocol. The area of application on the volar forearm between cubital fossa and wrist or on the intrascapular area on the back is marked using a felt-tipped marker pen, then stripped sequentially 20 times. This is accomplished by covering the 2.83 cm^2 treated with the precut tape, thus removing SC from a 6.45 cm^2 area, abundantly exceeding the area originally exposed in dosing. Constant and uniform pressure (100 g/cm^2) is applied on the tape for 5 s by resting an appropriate weight on the area, and the tape is then gradually removed from the skin in one draw. Each tape strip is stored individually in a 20 mL glass vial.

5 mL of concentrated nitric acid is added to the vials containing tape strips and decontamination swabs. After 3 h of vigorous agitation in a rotary shaker, the acid solution is diluted to 10 mL with deionized water prior to analysis by ICP-AES (Hostynek et al., 2001b).

22.4.2.3 *In Vivo* Diffusion of Metals Applied in the Elemental State

Micronized metal powder used (e.g., nickel or copper, 99.7% 3 μm particle size) is commercially available grade. The occlusive and semiocclusive application systems consist of a plastic chamber and Micropore semiocclusive (breathable) tape, respectively.

Polypropylene tape with a backing of pressure-sensitive acrylate adhesive tape is used for sequential SC removal by stripping. A 5% aqueous solution of Ethylene diamine tetraacidic acid (EDTA) (metal complexing agent) is used for skin decontamination prior to stripping. Scintillation vials containing tape strips in concentrated nitric acid are agitated over 4 h for extraction.

Prior to application of the metal powder, skin sites are cleansed with deionized water and dried using cotton swabs. The powder is applied on the volar forearm of volunteers between wrist and antecubital fossa. 25 mg of powder is placed on a 12 mm plastic chamber (1.15 cm^2), the chamber is then placed on the premarked area of the flexor surface of the arm, covered with transparent dressing, and is left undisturbed for the predetermined length of time. Treated areas are covered with a rigid, perforated plastic shield open at both ends to ensure free air circulation but at the same time preventing mechanical abrasion. The shield is held in place by taping it to the skin. At the end of the exposure period, the occlusive materials are removed, the site washed with a metal-complexing detergent containing EDTA using cotton swabs to remove all traces of metal left on the skin surface, then rinsed with De-ionized (DI) water. The skin is dried by tapping with cotton balls and finally by passing an air stream over the surface. The area of application is stripped sequentially 20 times after covering the 1.15 cm^2 treated area with a 1 $in.^2$ (6.45 cm^2) of the pre-cut adhesive tape strips, thus abundantly exceeding the perimeter of treated skin. Constant and uniform pressure (100 g/cm^2) is applied on the tape for 5 s, which was then gradually removed in one draw. The tapes with adhering SC are placed individually in scintillation vials, 5 mL of concentrated nitric acid (70%) is added and the vials agitated vigorously for 3 h on the rotary shaker. The acid solution is then diluted 20-fold with deionized water for ICP analysis (Hostynek et al., 2001a).

22.5 DIFFUSION DATA

22.5.1 Nickel

In industrialized parts of the world, nickel allergy is the leading cause of delayed as well as immediate type allergy following iatrogenic, respiratory, and gastrointestinal, as well as dermal exposure, with a constantly increasing prevalence (Hostynek et al., 2001a and references therein). The effects due to contact with the skin in particular are an indication of the formation of a skin-diffusible form of the metal apt to penetrate beyond the SC. This phenomenon was difficult to reconcile with earlier observations made when nickel diffusivity was investigated for its behavior in contact with the skin.

In *in vitro* tests with human skin, permeability coefficients for water-soluble inorganic nickel salts were measured in the range of 10^{-6}–10^{-4} cm/h, with lag times of up to 90 h preceding the appearance of the permeant in the acceptor phase (Table 22.1). This is difficult to reconcile with the phenomenon that even contact of intact skin with nickel-releasing metallic objects may elicit dermatitis in those allergic to the metal (Hostynek et al., 2001a and references therein). Evidence for a process that would explain the facile elicitation of contact allergy to nickel in particular was obtained through exposure of the skin of volunteers to the metal under occlusion. Using the technique of ICP-MS for skin strip analysis, it could be shown that at contact times as brief as 5 min with the metal under occlusion, nickel could be detected in the superficial strata of the SC, and that on longer exposure it had reached the live tissue of the epidermis (Hostynek et al., 2001a). Immunogenic nickel ion is likely to be formed *in situ* in reactions with skin exudates, to form small, hydrophilic complexes or salts, such as the chloride, pyruvate, or lactate of facile diffusion through the aqueous environment of sweat ducts. Lipophilic salts or soaps, in contrast, are likely to penetrate via the intercellular lipid bilayer (Hostynek, 2003).

22.5.1.1 Diffusion of Nickel Applied as Metal *In Vivo*

When exposure sites on the arm of volunteers were stripped 20 times for up to 96 h postdosing and the strips analyzed for metal content, the gradients of nickel distribution profiles increased proportionally with occlusion time. Although nickel values decreased from the superficial to the deeper levels of

TABLE 22.1
Diffusion of Nickel[a] through Human Skin *In Vitro*

Compound	$10^4 \times$ Kp (cm/h)	Comments	Reference
Sulfate	0.02–0.3	Epidermis	Samitz, 1976
Sulfate	0.03	Occluded	Fullerton, 1986
Sulfate	0.0085	Stratum corneum	Tanojo, 2001
Chloride	0.034	Full thickness	Fullerton, 1986
Chloride	0.53–2.3	Full thickness, occluded	Fullerton, 1986
Chloride	<0.045–0.37	Full thickness	Fullerton, 1988a
Chloride	0.55–1.4	Full thickness	Emilson, 1993
Chloride	0.07	Epidermis	Samitz, 1958
Chloride	0.46	Full thickness	Fullerton, 1986
Chloride	0.0068	Stratum corneum	Tanojo, 2001
Chloride	0.55	Full thickness	Emilson, 1993
Chloride	98[b]	Split thickness	Hostynek, 2003
Chloride	0.062[c]	Split thickness	Hostynek, 2003
Nitrate	0.0052	Stratum corneum	Tanojo, 2001
Acetate	0.0052	Stratum corneum	Tanojo, 2001
Di-octanoate	14[d]	Split thickness	Hostynek, 2003
Di-octanoate	0.09[e]	Split thickness	Hostynek, 2003

[a] Ni(II) compounds.
[b] Adolescent skin.
[c] Elderly skin.
[d] Adolescent skin.
[e] Elderly skin.

the SC, from the 10th strip to the 20th strip continued at constant levels.

Total nickel removed with 20 SC strips to the level of the glistening layer after maximum occlusion of 96 h was 41.6 mg/cm^2 (Hostynek et al., 2001a). The observations serve to confirm the function of the intercellular lipid matrix of the SC as alternate pathway for the diffusion of lipophilic nickel salts generated with skin exudates *in situ*. The role of skin as a toxicologically important route of exposure to xenobiotics in general and metal compounds in particular is thereby underscored, as altogether three routes of entry present themselves to potentially toxic agents.

22.5.1.2 Diffusion of Nickel Salts *In Vivo*

The characteristics of nickel penetration through the human skin *in vivo* were investigated by sequential stripping of the SC with adhesive tape following application of a number of soluble nickel salts. $NiC1_2{\cdot}6H_2O$, $NiSO_4{\cdot}6H_2O$, $Ni(NO_3)_2{\cdot}6H_2O$, and $(CH_3CO_2)_2Ni{\cdot}4H_2O$ were dissolved in methanol, the solvent chosen to minimize disruption of SC membrane integrity, optimize volatility, and thus expedite open application, as well as to attain adequate solubility of nickel salts. The depth–penetration profiles obtained by stripping of the previously occluded application sites on the arm and back of volunteers, at intervals of 30 min to 24 h postdosing, showed that up to 24 h most of the nickel remained on the surface or was absorbed in the uppermost levels of the SC; the concentration gradients varied with counter ion and anatomical site, dose, and exposure time. While the depth profiles converged toward nondetectable levels by the 20th strip for three of the salts, the levels for nitrate continued at low but constant levels toward the viable strata of the epidermis. Determined experimentally to be the least polar of the salts, the diffusion of the nitrate appears to be facilitated through the intercellular lipid domain of the SC (Hostynek et al., 2001b).

22.5.1.3 Diffusion of Nickel Salts *In Vitro*

Diffusion constants of nickel salts were measured through human SC. Aqueous solutions of $Ni(NO_3)_2$, $NiSO_4$, $NiCl_2$, and $Ni(\text{-}OOCCH_3)_2$ at 1% Ni^{2+} concentration were used as the donor solution, at 400 mL/cell. The receptor fluid, pure water, was collected up to 96 h after the application of the donor solutions. The nickel concentrations in the donor and receptor fluid, as well as in the SC were analyzed using ICP-MS (Tanojo et al., 2001).

Based on the total recovery of nickel from the experiments, about 98% of the dose remained in the donor solution, whereas 1% or less was retained in SC, and less than 1% was found in the receptor fluid. From the flux data, the steady-state permeability coefficients for nickel were calculated at 8.5 cm/h $\times 10^{-7}$ for the sulfate, 6.8 cm/h $\times 10^{-7}$ for the chloride, and 5.2 cm/h $\times 10^{-7}$ for both the nitrate and acetate.

22.5.1.4 Diffusion of a Nickel Salt versus a Nickel Soap *In Vitro*

In vitro results seemed to indicate increased diffusion of a relatively lipophilic nickel salt compared to more polar ones

(Hostynek et al., 2001b). The flux of nickel chloride, as prototype of a hydrophilic salt, was compared to that of nickel dioctanoate, a lipophilic, poorly water-soluble nickel soap, at comparable nickel concentrations in water using dermatomed skin. Based on the median steady-state nickel flux over 16 h, the permeability coefficients were

$$\mathrm{Kp} = 9.8 \times 10^{-3}\ \mathrm{cm/h} \pm 4.8 \times 10^{-3}$$

for nickel chloride, and

$$\mathrm{Kp} = 1.4 \times 10^{-3}\ \mathrm{cm/h} \pm 5.7 \times 10^{-4}$$

for nickel dioctanoate.

With a statistically significant difference, the unexpected slower diffusion of the dioctanoate as compared to the chloride can be ascribed to the molecular volume increment of the two octanoate groups, an effect that overrides the effect of polarity (Hostynek, 2003).

22.5.2 Copper

Since antiquity copper in several of its forms: pulverized or as sheet metal, as basic copper acetate (verdigris), or as copper sulfate (vitriol), has been used in the therapy of arthritic conditions. Modern science has recognized copper to be essential for most living organisms; playing a role in biochemical pathways and electron transport reactions in most living organisms.

Recognition that copper is an essential, homeostatically controlled trace element, whose concentration increases in serum and plasma of animals and humans as consequence of inflammation, as well as of its accumulation in inflamed joint exudates, led to the investigation of its therapeutic potential also as supplementary, exogenous agent in the treatment of numerous pathologies, and for the control of chronic inflammatory diseases. This resulted from investigations in animals showing that, while inflammation induces an increase in the amount of endogenous copper, exogenous sources can potentiate its anti-inflammatory (AI) effect in both acute and chronic models of inflammation (Milanino et al., 1993).

Ongoing investigations into the diffusivity of various copper compounds, peptides in particular, through human skin by quantitative *in vitro* experiments identified diffusion values (Kps), which may be adequate for application of those cupriphores as skin patches for AI therapy (Table 22.2).

22.5.2.1 Diffusion of Copper Applied as Metal *In Vivo*

The process of copper diffusion following application of the finely distributed (micronized) metal on the skin was demonstrated by applying the metal under occlusion and semiocclusion followed by SC stripping. Copper concentration profiles were recorded to the level of the living epidermal tissue. It thus became possible to document in depth penetration and presumed subsequent uptake by dermal microcirculation. Untreated skin of the volunteers was stripped in the same fashion for analysis, to determine baseline levels of this essential trace element prior to exposure.

TABLE 22.2
Diffusion of Copper[a] through Human Skin *In Vitro*

Compound	$10^4 \times$ Kp (cm/h)	Comments	Reference
Sulfate ($+ZnSO_4$)	0.032–0.045	Split thickness	Pirot et al., 1996
Sulfate	8.84	Split thickness	Unpublished[b]
Sulfate	0.027	Epidermis	Unpublished
Chloride ($+ZnCl_2$)	0.023–0.16	Split thickness	Pirot et al., 1996
Acetate	0.019	Split thickness	Unpublished
Acetate	0.066	Epidermis	Unpublished
Histidinate	1.03	Split thickness	Unpublished
Gluconate	2.28	Split thickness	Unpublished
Glycinate	24	Full thickness[c]	Walker et al., 1977
Glycinate	2.43	Split thickness	Unpublished
GHK[d]	0.76[e]	Split thickness	Unpublished
GHK	25.1[e]	Split thickness	Unpublished
GHK	0.002	Epidermis	Unpublished

[a] Cu(II) compounds.
[b] Unpublished data by Hostynek et al.
[c] Cat skin.
[d] Copper-Glycine-Histidine-Lysine.
[e] Kps from separate sets of cells in the same diffusion experiment.

Under occlusion thus excluding access of air, up to 72 h copper values decreased from the superficial to the deeper layers of the SC with gradients increasing commensurately with occlusion time, characteristic of passive diffusion processes. From the 10th strip on, however, levels reverted to background values.

Under semiocclusion, allowing access of oxygen by covering the applications with "breathable" tape, initial copper values lay significantly above baseline values and concentration gradients increased proportionally with occlusion time. At 72 h exposure, from the 10th to the 20th strip reaching the glistening epidermal layer, copper values continued at constant levels, significantly above baseline values. At 72 h, an average of about 0.6 mg/cm^2 of copper was measured in each of the layers going from 15 to 20 (Hostynek, unpublished data).

The results indicate that, in contact with skin, copper will oxidize and may penetrate the SC after forming an ion pair with skin exudates. The rate of reaction seems to depend on contact time and availability of oxygen. Also a marked interindividual difference was observed in baseline values, and subsequently in the amounts of copper absorbed.

It is also worth noting that, in contrast to organic compounds (e.g., drugs) for which accelerated absorption under occlusive conditions is predictable due to increased SC hydration, increased blood flow and temperature (Zhai and Maibach, 2001), this does not necessarily apply to all metals, for which exclusion of oxygen can have the opposite effect. In the case of nickel, described earlier, corrosivity permits oxidation of the metal even under limited access of oxygen, this in contrast to copper, which has a higher ionization potential or resistance to oxidation.

22.5.2.2 Diffusion of Copper Compounds *In Vitro*

Work linking copper and inflammation has been done by Sorenson, who demonstrated that copper(II) acetate alone has an AI activity in experimental models of inflammation, with the suggestion that the active metabolites for AI activity are copper chelates (Sorenson, 1982a,b; Sorenson and Berthon, 1995). Sorenson and Hangarter (1977) then reported on the AI effectiveness of injectable low molecular weight copper complexes in the treatment of rheumatoid arthritis (RA) and other acute and chronic degenerative diseases in some 1500 patients over 30 years. It appears important to maintain a needed minimum level of copper in tissues for the control of inflammation (Milanino et al., 1978, 1985).

These observations were pivotal in motivating the search for skin-diffusible forms of copper toward administration of exogenous copper, as topical cupriphores represent a potential alternative to AI therapy.

The current investigations into the diffusivity of certain copper compounds, peptides in particular, through human skin by quantitative *in vitro* experiments identified diffusion values (Kps), which may be adequate for application of those cupriphores as skin patches for AI therapy (Table 22.2).

22.6 DISCUSSION AND CONCLUSIONS

The process of barrier diffusion by metal ions is multifactorial, and the rate of their diffusion imponderable, both *in vitro* and *in vivo* due to a number of potentially promoting and retarding processes simultaneously involved.

Although there is a considerable volume of literature addressing skin absorption of metal compounds, the data vary considerably from experiment to experiment. Results are difficult to reconcile due to the diversity of conditions used: diffusion cell design, vehicle, metal speciation, skin tissue compartment, anatomical site, human versus animal tissue, data acquired *in vivo* versus *in vitro* etc., some of which have been briefly discussed earlier. Results in the research of skin diffusion by metal compounds are too fragmented to make possible the derivation of a unifying algorithm with which to assess dermal absorption of metal compounds *a priori*, or to bring results from different researchers in relation to each other. In determining skin diffusivity of metals *in vitro* for toxicological risk assessment, for instance, it would appear important not to limit the focus solely on permeant reaching the receptor phase. Analysis should also include permeant retained in the strata of the skin for topical bioavailability purposes. In the case of some metals, the more electrophilic ions in particular, that value may be the preponderant quantity, potentially a significant factor for consideration in exposure risk analysis. As an example serves the apparent paradox of nickel diffusion—measured *in vitro*—it appears extremely slow, and yet *in vivo*, it elicits allergic skin reactions on mere contact; nickel retained in the epidermis appears to be a determinant for immune response. Also, metals of slow diffusivity such as transition metals encounter a countercurrent effect in the strata of the skin. As the barrier is characterized by continuous desquamation with a total turnover over 2–3 weeks (corresponding to an approximate "inverse" Kp of 10^{-6}), bioavailability of the metal may be less than what is indicated by absorption in skin tissue, since epidermal turnover can significantly reduce further diffusion into the systemic circulation of highly electrophilic compounds. Sweat can also be such a contributing countercurrent factor to the loss of water-soluble metal salts diffusing into sweat ducts. Some metals form a reservoir that can be mobilized on homeostatic demand, such as copper, zinc, or calcium; others again penetrate into the strata by passive diffusion and, albeit slowly, reach the systemic compartment. At times the same metal can follow several patterns. Data, which deduct Kps from values reaching the receptor compartment only may underestimate the true level of absorption. Seen from the perspective of the presently available database, derivation of a unifying algorithm predictive of dermal absorption of metal compounds does not appear feasible.

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23 Chemically Induced Scleroderma

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23.1 INTRODUCTION

Exposure to environmental, occupational, and iatrogenic chemicals may sometimes be followed by scleroderma-like clinical findings whose cause and effect relationship is not easily established. This chapter reviews some chemicals that appear to suggest an association between chemical exposure and subsequent symptomatology suggestive of scleroderma.

23.2 ENVIRONMENTAL AGENTS

23.2.1 Toxic Oil Syndrome

In 1981, an epidermal multisystem disorder with chronic scleroderma-like cutaneous changes was linked with the ingestion of rapeseed oil that was sold as olive oil in Spain. Called the toxic oil syndrome, more than 300 deaths resulted (Phelps and Fleishmajer, 1988). The patients first presented with an acute phase of fever, pneumonitis, adenopathy, nausea, arthralgias, and pruritic exanthems (Martinez-Tello et al., 1982). Eosinophilia was always present. The initial symptoms soon resolved, and an intermediate phase of intense myalgias, edema, and paresthesias of the extremities occurred. Months later, many patients, especially women, had progressive neuromuscular atrophy and paralysis, pulmonary hypertension, sicca syndrome, Raynaud's phenomenon, and alopecia. The pruritic exanthems became sclerotic, leading to a diffuse scleroderma-like picture (Martinez-Tello et al., 1982). The pruritic exanthem resembled those caused by viruses and involved the abdomen, trunk, and limbs. Sometimes there was also an associated erythema multiforme or palpable purpura. Another skin eruption occurred in 10% of patients that consisted of yellowish or brownish papules, which spared the palms and soles (Iglesias and De Morgas, 1983).

The histopathologic features have been well characterized and are caused by a multiorgan systemic vasculopathy. The chronic dermal lesions showed thickening of the collagen bundles, hyalinization, perivascular inflammation, endothelial swelling, and slight luminal obliteration (Martinez-Tello et al., 1982; Fonsecaea and Soils, 1985). Toxic substances in the oil (e.g., anilides) may stimulate the proliferation of mast cells that thereby interact with fibroblasts and mediate sclerosis (Prestana and Munoz, 1982).

Others have suggested that the progressive vasculopathy primarily stimulates fibrosis, including the sclerotic changes observed clinically (Martinez-Tello et al., 1982). Soon after the initial cases of toxic oil syndrome were reported, more than 3 million liters of oil were seized; however, the causative agent may be persistent in stored oils because it appears to be stable (Phelps and Fleishmajer, 1988). The contaminant found in the toxic oil has been identified as 3-phenylamino-1, 2 propanediol (D'Cruz, 2000).

23.2.2 Hexachlorobenzene

During the outbreaks of porphyria turcica due to accidentally ingested hexachlorobenzene-treated seed grain, patients were also noted to have sclerodermoid changes (Peters et al., 1982; Schmid, 1960; Cripps et al., 1980). It is believed that these sclerodermoid changes may have been due to the production of hyalin stimulated by the deposition of porphyrins in the skin (Cripps et al., 1980). The pathogenesis of the sclerodermoid changes in porphyria turcica may be related to the dermal perivascular hyalinization as a residual alteration of the abnormal accumulation of porphyrins (Torinuki et al., 1989). Hexachlorobenzene is no longer produced for use as a fungicide. It is now present only as an unwelcomed byproduct in the manufacturing of pesticides and hydrocarbons (Burns et al., 1974; Peters et al., 1982). Hexachlorobenzene has a strong tendency to persist in soil and to accumulate in aquatic environments (Isensee et al., 1976).

23.2.3 Urea Formaldehyde Foam Insulation

Urea formaldehyde foam insulation may lead to a scleroderma-like syndrome that has been reported in one case. The patient, shortly after cleaning out the ceiling of his garage, developed Raynaud's phenomenon, arthritis, pulmonary fibrosis, and sclerodermatous skin changes. Dermal fibrosis was noted histologically. It is thought that urea formaldehyde foam insulation may elicit an immunologic response, which, in conjunction with genetic susceptibility, may lead to this syndrome (Rush and Chaiton, 1986). However, as only one case has been reported, chance association remains a very likely explanation for its appearance.

23.3 OCCUPATIONAL AGENTS

23.3.1 Vinyl Chloride Disease

Some workers in the plastics industry, especially those exposed for long periods to the unreacted vinyl chloride monomer while cleaning polymerization reactors, have had a scleroderma-like illness (Meyerson and Meier, 1972). This is characterized by fatigue, Raynaud's phenomenon, shortened fingers with sclerodactyly, distinctive distal phalangeal bone resorption sparing the tufts (acro-osteolysis), facial telangiectasias, and pulmonary and hepatic fibrosis. Angiosarcoma can develop in areas of severe hepatic involvement with the added association of ethanol ingestion. The findings in the skin include plaque-like fibrotic lesions that show thickened dermal collagen histologically with perivascular lymphocytic infiltrates. Endothelial cell swelling has also been seen in the microvasculature (Meyerson and Meier, 1972; Maricq et al., 1976; Steen 1999).

Because less than 10% of those exposed are susceptible, immunogenetic factors appear to be additionally important in providing for the capillary pathologic condition that may mediate vinyl chloride disease (Ward et al., 1976). Cellular immune reactivity was decreased, while circulating immune complexes and human leukocyte antigen (HLA) B8, and DR3 frequency were increased in workers who experienced vinyl chloride disease (Meyerson and Meier, 1972; Veltman, 1980; Black et al., 1983).

A genetic susceptibility to developing scleroderma when exposed to vinyl chloride may be a possible reason for the great difference in the reported incidences of the disease in vinyl chloride workers from various countries. For example, Czirjak has pointed out that British vinyl chloride workers have a much higher frequency of developing scleroderma compared to Hungarian workers (Czirjak et al., 1995).

The vascular occlusion, in particular, may stimulate new collagen synthesis through ischemia, thus producing fibrosis (Black et al., 1983). However, if the level of exposure to vinyl chloride monomer is reduced in the workplace, additional cases of vinyl chloride disease are less likely to occur (Maricq et al., 1976).

23.3.2 Trichloroethylene and Perchloroethylene

Chemically similar to vinyl chloride, trichloroethylene and perchloroethylene exposure may also lead to scleroderma-like conditions. Trichloroethylene is used as a degreasing agent, which is used in clothes dry-cleaning. Upon exposure to trichloroethylene, a severe irritant contact dermatitis may occur. Characteristic features of the scleroderma-like condition produced by trichloroethylene include myalgia, weakness, hepatitis, Raynaud's phenomenon, and sclerodermoid skin tightening (Sparrow, 1977). Other symptoms that have been reported associated with prolonged trichloroethylene exposure include malabsorption syndrome, pigmentation, gynecomastia, impotency, lymphadenopathy, peripheral neuropathy, and sleepiness (Saihan et al., 1978).

Although one series of workers who developed scleroderma usually involved prolonged exposure to trichloroethylene in the range of 2–14 years, one woman reportedly developed acute swelling of her hands and scleroderma after only 2.5 h of intense exposure to the chemical (Bottomlay et al., 1993). A study by Nietert and associates demonstrated

a statistically significant association between organic solvents including trichloroethylene with men who had scleroderma and positivity for antitopoisomerase antibody. This association may have implications concerning the pathogenesis of scleroderma in some patients (Nitert et al., 1998). Trichloroethylene has also been implicated as a cause of localized morphea of the forearm and ankles in a female worker who was exposed to it by inhalation (Czirják et al., 1994).

Skin biopsy in trichloroethylene exposure reveals extensive dermal fibrosis extending into subcutaneous tissue consistent with scleroderma. Although sclerodermoid changes are not pathologically demonstrated in perchloroethylene exposure, endothelial swelling and a perivascular infiltrate have been noted (Sparrow, 1977).

The pathogenesis of trichloroethylene and perchloroethylene disease may involve the altering of proteins by these chemicals, providing for an immune response to be manifested in genetically susceptible individuals (Sparrow, 1977; Lockey et al., 1987; Khan et al., 1995).

23.3.3 Aromatic Hydrocarbon Solvents

Aromatic hydrocarbon solvents such as benzene and toluene, which are used for cleaning clothes, painting, and removing oil and grease in industry, have been associated with scleroderma-like features (Walder, 1983). These chemicals predominantly enter the body through vapor inhalation and later produce systemic manifestations. Scleroderma skin changes are limited only to areas of direct contact (Haustein and Ziegler, 1982). Aliphatic hydrocarbons may also produce scleroderma changes through either vapor exposure or direct contact (Yamakage and Ishikawa, 1982). Histologic features are consistent with scleroderma.

23.3.4 Organic Solvents

A study of 21 women with systemic scleroderma revealed that eight of them were exposed to organic solvents on an occupational level (Czirják et al., 1987). Other abnormalities reported in these eight patients included hypofunction of the thyroid gland, delayed-type hypersensitivity to D-penicillamine, antimicrotubullis antibody, and lupus anticoagulant. This study also reported that there was a slight decrease in OKT4 positive cells in the peripheral blood of the eight affected patients.

A case reference study of 21 cases of scleroderma in the province of Trento, Italy, concluded that there was a statistically significant association between organic solvent exposure and the development of scleroderma. The range of exposure time to the solvents was 9–38 years (Bovenzi et al., 1995).

The toxicity of aliphatic hydrocarbons was further demonstrated by the induction of skin changes through intraperitoneal injection in mice (Haustein and Ziegler, 1982). Solvents are constituents of paints, varnishes, lacquers, polishes, inks, adhesives, pharmaceutical products, and preservatives. If protective clothing is worn and ventilation is improved, thereby decreasing contact, disease production should be limited (Haustein and Ziegler, 1982).

Another report suggests that prolonged exposure to toluenes, toluidines, xylenes, xylidines, aniline compounds, and ethanolamine and its derivatives produced a syndrome of cold receptivity, restrictive lung defect, peripheral neuropathy, esophageal dysfunction, hypertension, monoclonal paraproteinemia, and diffuse thickening of the skin and subcutaneous tissue, which spared the hands and feet (Bottomlay et al., 1993).

23.3.5 Epoxy Resins

In the early 1980s, 6 of the 233 Japanese workers exposed to the vapor of epoxy resins used in television set transformer production were reported to have experienced fatigue, myalgia, arthralgia, erythema, and skin sclerosis (Yamakage et al., 1980). The cause was ascertained to be the amine known as *bis*(4-amino-3-methyl-cyclohexyl)methane. Histologically, dense collagen bundles were noted in the lower dermis with superimposed glycosaminoglycan deposits and a perivascular lymphocytic infiltrate. Investigators found the full histologic picture to be more consistent with that of generalized morphea than systemic scleroderma (Yamakage et al., 1980).

Heparan sulfate extracted from the glucosaminoglycans in the skin of these patients induced sclerotic changes in mice, indicating that, perhaps through faulty metabolism, the biogenic amine in eposy resins indirectly elicits the clinical syndrome observed (Ishikawa et al., 1980). Further outbreaks of epoxy resin induced scleroderma have not been reported, indicating that the toxic exposure to this amine may be an incidental finding (Yamakage et al., 1980).

23.3.6 *meta*-Phenylenediamine

Workers exposed to *meta*-phenylenediamine have been reported to develop a systemic sclerosis-like disorder. These workers developed Raynaud's phenomenon, hyperpigmentation, pulmonary fibrosis, and sclerodermatous skin changes (Owens and Medsger, 1988). *meta*-Phenylenediamine, as a biogenic amine, most likely induces sclerodermatous changes similarly to the epoxy resins. Chemical workers exposed to large quantities appear to be at most risk.

23.3.7 Detergents

One report from Japan suggests that a patient exposed to a commercial detergent containing polyoxyethylene alkyl ether and fatty acid alkanol amide developed Raynaud's phenomenon, joint pain and decreased ability to close his hands within 3 months after exposure (Tanaka et al., 1993). By 13 months after exposure, he had hardening of the skin of his trunk as well. A skin biopsy done 10 months after exposure was consistent with scleroderma.

23.3.8 Silica

Exposure to silica dust, primarily in mining in South Africa, Pennsylvania, and Germany, has not been linked with pseudoscleroderma, but with true progressive systemic sclerosis (Rodnan et al., 1967; Reiser and Last, 1979). Symptoms

include Raynaud's phenomenon, arthritis, sclerodactyly, and other classic features of scleroderma. Silicotic lung changes frequently precede the scleroderma in those patients who are affected (Rodnan et al., 1967; Reiser and Last, 1979). The pulmonary pathologic condition is consistent with progressive systemic sclerosis showing diffuse alveolar, interstitial, perivascular, and vascular fibrosis (Reiser and Last, 1979).

The role of silica in the pathogenesis of scleroderma is thought to involve the enhancement of fibroblast collagenosis, perhaps by immune adjuvantcy (Rodnan et al., 1967; Reiser and Last, 1979). Whether this stems from macrophage fibroblast interaction remains debatable.

It has been proposed that the mechanism of action of silica exposure producing progressive systemic sclerosis involves the macrophages engulfing the silica, then as the macrophages die the silica is released again. IL-1 is then released which stimulates fibroblasts to increase their production of collagen. This increased collagen production leads to skin sclerosis, vascular occlusion, and fibrosis of the lungs (Yañez et al., 1992). On a basic research level, Haustein and Anderegy have been able to demonstrate that silica when injected into mice can produce changes in fibroblasts and mononuclear cells, which are similar to changes seen in the cells in patients with idiopathic scleroderma (Haustein and Anderegy, 1998). Studies have shown that exposure to silica can lead to the production of antinuclear antibodies, immune complexes, and damage to endothelial cells (Rustin et al., 1990). The pathogenicity of silica *in vitro* and *in vivo* is related to its structure, particle size, and concentration, and its association with scleroderma is less well established than its relationship with lung disease (Rodnan et al., 1967; Reiser and Last, 1979). According to investigators, the incidence of progressive systemic sclerosis in Germany is 25 times higher in workplaces where there is exposure to silica and 110 times higher for those patients with silicosis. This indicates that some immunomodulating mechanism may be operating in an adjuvant manner (Rodnan et al., 1967; Reiser and Last, 1979).

A study by McHugh et al. demonstrated increased frequency of antitopoisomerase antibody levels in patients with scleroderma who were exposed to silica (McHugh et al., 1994). Similarly, another study (Conrad et al., 1995) showed an increased frequency of positive anticentromere antibody levels in men who developed scleroderma who were exposed to silica. This study also surprisingly demonstrated that there were increased anticentromere antibodies even in men who were exposed to silica at high levels while working in uranium mines and did not develop scleroderma (Conrad et al., 1995).

23.3.9 Pesticides

Sclerodermatous changes have occurred in workers handling pesticides such as chlordane, heptachlor, malathion, parathion DDT, sodium dinitro-*ortho*-cresolate, and 7-chlorocylohexane. Reported patients developed Raynaud's phenomenon and sclerodermatous skin changes, but no internal involvement. Histological features are compatible with scleroderma (Jablonska, 1975).

There is a report of a 58-year-old West Indian man who had a 3-month intense exposure to a herbicide, which contained bromacil, diuron, and aminotriazole and then developed generalized sclerosis of his skin along with sclerodermatous symptoms of his esophagus and muscles (Dunnill and Black, 1994). The patient suffered particularly from prominent acrosclerosis of his hands, which extended up his forearms. It should be noted that aminotriazole has also been reported to produce an allergic contact dermatitis in workers exposed to it (English et al., 1986).

23.4 IATROGENIC AGENTS

There have been several reports of scleroderma developing between 2 and 21 years after silicone breast implantation (Sahn et al., 1990). Silicone fluids in breast implants are chemically known as dimethylpolysiloxane. These substances can "bleed" through the silicone envelope into surrounding tissue.

Clinical symptoms include Raynaud's phenomenon, sicca syndrome, pulmonary and gastrointestinal abnormalities, and diffuse skin sclerosis that usually begins in the upper extremities (Lilla and Vistner, 1976; Kumagai et al., 1984; McCoy et al., 1984; Brozenza et al., 1988; Silverstein et al., 1988; Spiera, 1988). With injection, breast masses, local erythema, and adenopathy may develop locally (McCoy et al., 1984). Histopathologic examination shows thickening of collagen bundles through the dermis, sclerosis of subcutaneous fat, blood vessel dilatation, and a perivascular lymphocytic infiltrate (Lilla and Vistner, 1976; Kumagai et al., 1984; McCoy et al., 1984; Brozenza et al., 1988; Silverstein et al., 1988; Spiera, 1988; Varga et al., 1989).

It has been proposed that macrophages ingest the silicone and produce silica. The presence of silica would then stimulate a more intense immunologic reaction. This would further stimulate macrophages to release transforming growth factor-β and platelet-derived growth factor, which would then stimulate fibroblasts to produce collagen (Sahn et al., 1990). Upon removal of the breast implants, some, but not all, patients show gradual improvement in the edema and firmness of their skin.

23.4.1 Bleomycin and Cisplatin

Scleroderma-like changes have been reported in patients with cancers of primary gonadal origin after treatment with bleomycin and cisplatin (Nixon et al., 1981; Finch et al., 1980). Histopathologically, in bleomycin-induced scleroderma, the dermis shows dense collagen with areas of homogenization, particularly associated with endothelial thickening (Cohen et al., 1973).

Many factors may contribute in the development of this iatrogenic syndrome. Bleomycin produces endothelial cell injury by peroxidation of the plasma membrane, which may then initiate fibrosis (Adamson and Bowden, 1974; Burkhardt and Holtje, 1976; Tom and Montgomery, 1980; Mountz et al., 1983). Bleomycin has stimulated collagen synthesis by

cultured normal skin fibroblasts, and may induce a lymphoproliferative response *in vitro* (Mountz et al., 1983).

There is a case report describing three patients having been exposed to less than a total of 100 U total of bleomycin, developing Raynaud's phenomenon along with thickening of fingers, periungual erythema, and sclerodactyly all within 6 months of exposure, some as early as 3 months after exposure (Kerr and Spiera, 1992).

23.4.2 Pentazocine

Pentazocine, when used as an analgesic, has been linked to the development of diffuse pigmentation changes and sclerotic fibrosis around injection sites, with the formation of large, irregular ulcers (Parks et al., 1971; Swanson et al., 1973; Palestine et al., 1980). On examination of a skin biopsy specimen, there is dermal thickening and fibrosis with an inflammatory cell infiltrate that is granulomatous in areas. Histologic examination also shows both venous and arterial small vessel thrombosis, endarteritis with endothelial hyperplasia, and lymphohistiocytic perivascular inflammation.

Pentazocine-induced fibrosis has been related to vascular ischemia, and the resultant tissue changes to pentazocine-induced vasoconstriction. Because their vasculature is already compromised, patients with diabetes may be more prone to developing these skin changes with the additive effect of pentazocine-induced vasoconstriction (Parks et al., 1971; Swanson et al., 1973; Palestine et al., 1980).

23.4.3 Ethosuximide

Ethosuximide, an anticonvulsant, has been associated with a lupus-scleroderma syndrome in one patient (Teoh and Chan, 1975). Clinical features included fevers, arthritis, malar flush, maculopapular eruption, and widespread skin sclerosis. Histological examination revealed dermal collagen bundles to be homogeneous and hyalinized with a perivascular lymphocytic infiltrate (Teoh and Chan, 1975).

An immunopathogenetic mechanism appears evident in ethosuximide-induced scleroderma with the coincident development of lupus. Connective tissue changes may also be considered when evaluating epileptic patients with rheumatological symptoms, as well as primary connective tissue diseases (Alarcon-Segovia, 1969; Teoh and Chan, 1975).

23.4.4 Penicillamine

A teenager with Wilson's disease who was treated with penicillamine developed systemic sclerosis-like lesions. Hyperpigmentation, pulmonary restriction, and proximal scleroderma were also evident (Miyagawa et al., 1987).

Penicillamine has been noted to have connective tissue and autoimmune effects that may involve its relation to the development of a scleroderma-like picture (Fulghum and Katz, 1968; Hasimoto et al., 1981; Walsh, 1981). As penicillamine is utilized as a treatment for progressive systemic sclerosis, its "sclerodermatous" effect is questionable in consideration of this one case.

There has also been a case of morphea-like plaques developing in a patient with rheumatoid-type arthritis who had been treated with D-penicillamine at 250 mg/day for 1 year (Bernstein et al., 1981).

23.4.5 Ergot Methysergide

Patients with migraine headaches treated with ergot and methysergide have been linked with the development of scleroderma. Clinical manifestations noted included Raynaud's phenomenon, as well as additional features associated with progressive systemic sclerosis. Histological features were also consistent with scleroderma (Robb, 1975).

As similar vasoactive phases are involved in the pathogenesis of migraines and Raynaud's phenomenon, it would seem as though they could be linked. Perhaps ergot and methysergide perpetuate this linkage by mediating vascular hyperreactivity leading to a scleroderma-like condition (Barret and McSharry, 1975; Robb, 1975). Ergot, in particular, has been shown to exacerbate Raynaud's phenomenon, and caution may be needed in treating patients with migraine headaches with these medications (Barret and McSharry, 1975; Robb, 1975; Goldberg et al., 1978).

23.5 NONPRESCRIPTION DRUGS

23.5.1 Eosinophilia-Myalgia Syndrome

First reported in October 1989 by the Centers for Disease Control and now including more than 1500 cases, an association has been made between the ingestion of L-tryptophan and the occurrence of the eosinophilia-myalgia syndrome, which often involves sclerodermoid features (Hertzman et al., 1990; Silver et al., 1990). L-tryptophan is an essential amino acid that is available in dietary supplement form. It is used to treat insomnia, depression, tinnitus, and premenstrual-related symptoms. The eosinophilia-myalgia syndrome is primarily defined by severe, incapacitating myalgias and peripheral eosinophilia (Sternberg et al., 1980; Centers for Disease Control, 1990a,b; Hertzman et al., 1990; Silver et al., 1990; Stutsker et al., 1990; Varga et al., 1990).

Characteristic clinical features also include arthralgias, fevers, dyspnea, edema, and macular exanthems, followed by acrally sparing sclerodermatous induration with a puckered or a peau d'orange appearance (Fishman and Russo, 1991). Eosinophilic fasciitis and papular mucinosis may also be observed (Lin et al., 1992). Raynaud's phenomenon, however, is absent in this condition (Sternberg et al., 1980; Centers for Disease Control, 1990a,b; Hertzman et al., 1990; Silver et al., 1990; Stutsker et al., 1990; Varga et al., 1990). On deep incisional biopsy, dermal thickening and homogenization of collagen bundles with mucin accumulation are seen replacing fat and adnexa. Blood vessel walls show thickening and endothelial swelling, and mast cells and plasma cells may be seen perivascularly and in the dermal infiltrate (Lin et al., 1992). Two additional observations are minimal tissue eosinophilia, despite the extent of peripheral eosinophilia, and minimal myofiber atrophy, regeneration, or

necrosis, despite the clinically significant myalgias (Kaufman et al., 1990; Silver et al., 1990; Lin et al., 1992).

The fibrotic effects of L-tryptophan have most often been associated with its role as a precursor in two metabolic pathways, one involves the production of serotonin and the other involves the production of nicotinic acid (Hertzman et al., 1990; Silver et al., 1990). This is supported by the development of cutaneous sclerosis in the carcinoid syndrome in which serotonin is produced in excess (Zarafonetis et al., 1959). The kynurenine pathway leads to the production of nicotinic acid. A patient with progressive systemic sclerosis has been described who had an elevated urinary kynurenine level along with a partial deficiency of kynurenine hydroxylase (Zarafonetis et al., 1959; Conolly et al., 1990). Additionally, in a patient who received L-5-hydroxytryptophan and carbidopa, both pathways may have been involved in the production of scleroderma because carbidopa can bind pyridoxal phosphate, which is a necessary cofactor in each pathway (Zarafonetis et al., 1959; Conolly et al., 1990). Isoniazid has been reported to have produced an apparent pyridoxal phosphate deficiency with elevated levels of urinary kynurenine and pellagra, as well as subsequent sclerodermatous changes (McConell and Cheltham, 1952). Some investigators report a possible genetic mechanism for L-tryptophan-induced fibrosis. *In situ* hybridizations have shown L-tryptophan to enhance expression of the collagen gene resulting in dermal and fascial fibrosis (Price et al., 1967). Mast cells may mediate this interaction (Lin et al., 1992; Conolly et al., 1990). Finally, endothelial changes with associated vascular alterations as observed histologically may induce sclerosis (Lin etal., 1992). Tryptophan is no longer widely available because it was taken off the market in November 1989 by the Food and Drug Administration. Factors such as individual susceptibility and particular preparation contaminants may explain the relatively low incidence of eosinophilia-myalgia syndrome (Centers for Disease Control, 1989). However, recent data appear to implicate a Japanese company that also produces the presumed toxin di-L-tryptophan as the source of the L-tryptophan-producing eosinophilia-myalgia syndrome (Stutsker et al., 1990).

The contaminant responsible for eosinophilia-myalgia syndrome appears to have been 3-(phenylamino) alanine (D'Cruz, 2000). Philen and Hill (1993) have pointed out that the contaminants in toxic oil syndrome and eosinophilia-myalgia syndrome are chemically similar and this may explain why the two syndromes produce similar clinical features.

23.5.2 Appetite Suppressants

A report out of England has proposed that the appetite suppressants diethylpropion and mazindol may induce sclerosis. Patients had taken appetite suppressants and subsequently developed Raynaud's phenomenon, arthritis, pulmonary and gastrointestinal symptoms, and sclerodermatous skin changes (Tomlinson and Jayson, 1984). Histological examination was not performed.

The sympathomimetic properties of diethylpropion may lead to vasomotor stress, which leads to Raynaud's phenomenon and other features of skin ischemia. Diethylpropion and mazindol also have serotonergic properties that may play a role in the kynurenine and serotonin metabolism reported in other scleroderma-like conditions (Bourgeois and Aeschlimann, 1991).

23.5.3 Cocaine

The association between cocaine abuse and scleroderma was suggested in two male patients (Trozak and Gould, 1984; Kerr, 1989). Common features included Raynaud's phenomenon, acral scarring, and diffuse sclerosis (Trozak and Gould, 1984). A skin biopsy specimen of one of the patients showed dermal thickening and collagen sclerosis.

An association between cocaine use and sclerodermatous changes has also been reported in four other young males (Kilaru and Kim Sequeira, 1991). All cases showed Raynaud's phenomena, sclerodactyly, skin thickening proximal to metacarpal phalangeal joints, and digital scars. It was hypothesized that the vasoconstrictive properties of cocaine may either promote or unmask an underlying propensity for scleroderma. Skin biopsy of one of the four patients demonstrated dermal thickening and collagen sclerosis (Kilaru and Kim Sequeira, 1991).

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24 Chemical Agents That Cause Depigmentation

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24.1 INTRODUCTION

Many disorders result in disturbances of pigment formation by the melanocytes (Mosher et al., 1987). Hypomelanosis or a decrease in the formation of the pigment melanin may be caused by many disorders. Leukoderma, derived from the Greek terms, λευκο white + δερμα skin, due to chemical exposure has been associated with several different classes of compounds, most being phenols or thiols. These chemicals are useful as antioxidants and find utility in rubbers and plastics, in foods, and as polymerization inhibitors in monomers. Because of the widespread use of these chemicals, it is important to examine the effects of exposure and the mechanism of depigmentation.

24.2 HISTORY

Occupational leukoderma due to exposure to chemicals was first reported more than 65 years ago (Oliver et al., 1939). The depigmentation, which may resemble vitiligo, was produced by the monobenzyl ether of hydroquinone (MBEH), which translocated from rubber gloves worn by workers. Once it was documented that chemical agents could depigment the skin, it became important to test them for this property, and several laboratory procedures have been developed for this purpose. The methods used for testing depigmenting chemicals have been reviewed previously (Gellin and Maibach, 1987).

24.3 CHEMICAL STRUCTURES CAUSING DEPIGMENTATION

Chemical depigmentation has been associated with a variety of compounds. Most are phenols or sulthydryl compounds, but divalent metals that bind to melanin have also been implicated. These materials are useful as antioxidants and inhibitors of polymerization. Because of these properties, they are employed in a wide variety of products and can potentially contact many people during manufacture and use. In addition, some of these agents have been applied intentionally for the purpose of lightening hyperpigmented skin. The structures and acronyms of these materials are shown in Figure 24.1, but catechol (CAT) and phenol have not been included. MBEH has been used to intentionally depigment hyperpigmented skin in humans (Lerner and Fitzpatrick, 1953; Becker and Spencer, 1962). The results were not very satisfactory because the response had wide individual variation. Furthermore, depigmentation occurred at sites remote from the site of application. There was no depigmentation without some evidence of inflammation. In another study, MBEH was used at 10–33% concentration in lotions and ointments, and was deemed to give satisfactory results when used to treat hyperpigmentation in patients (Denton et al., 1952). Bleaching creams containing hydroquinone (HY) have also been reported to cause leukoderma (Fisher, 1982).

Some clinical data have been gathered from exposures to products containing depigmenting chemicals. Some ceramic lacquers contain phenolic compounds (exact structure unidentified), and one case of leukoderma has been reported from exposure to these materials (Tosti et al., 1991). One case of leukoderma from contact with neoprene swim goggles has been reported (Goette, 1984), but the agent responsible was not identified. A case of hypopigmentation due to contact with phototypesetting paper containing *tert*-butyl catechol (TBC) has been described (Fardal and Gurphey, 1983). TBC is also used as an antioxidant in industrial lubricants and workers who come in contact with these experience depigmentation (Gellin et al., 1970). Antioxidants are added to polyethylene film, and these materials can translocate if the film is in contact with skin. Polyethylene film, used as an occlusive dressing during steroid treatment, produced two cases of leukoderma (Vollum, 1971). A case of depigmentation due to adhesive

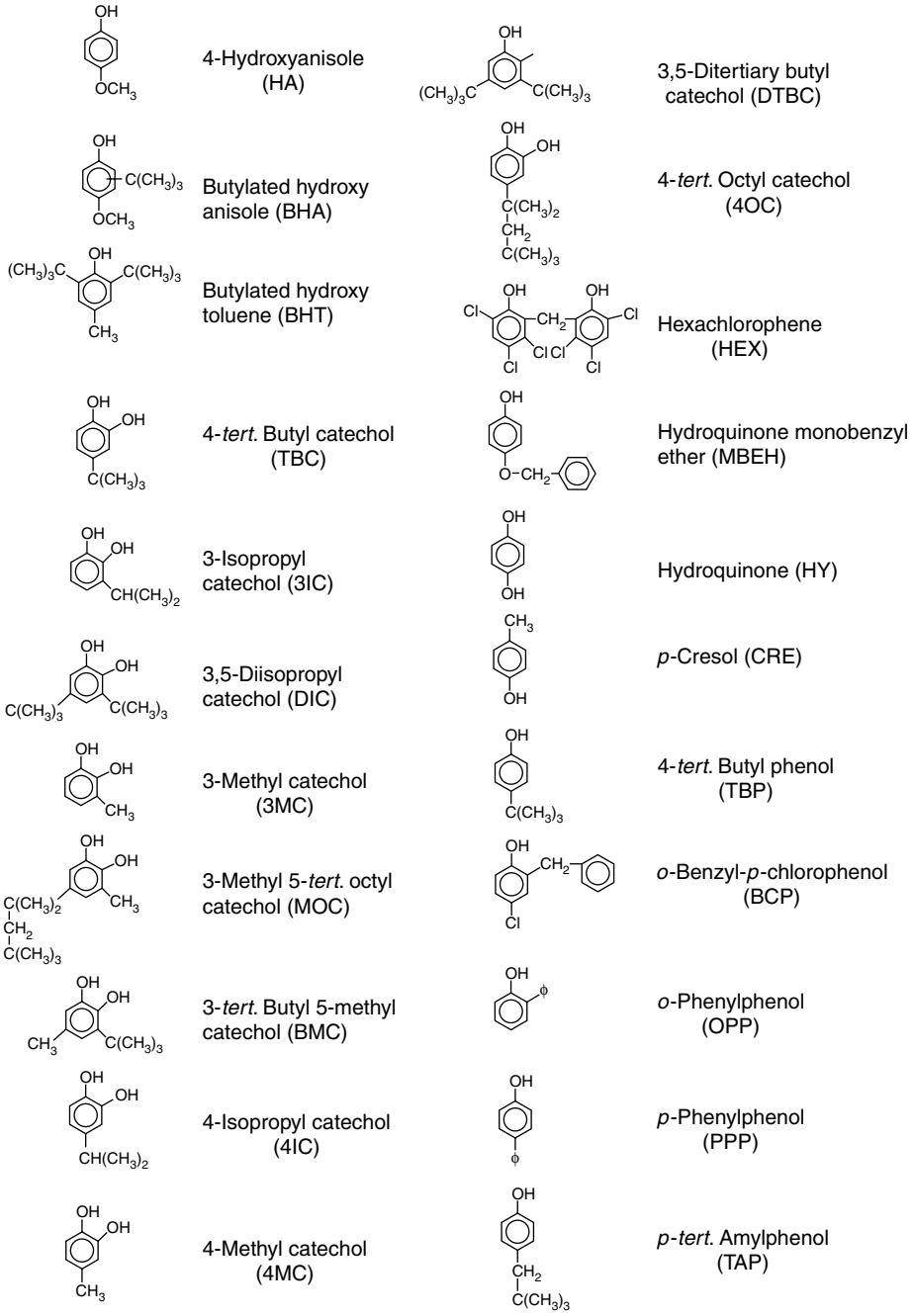


FIGURE 24.1 Compounds that have caused depigmentation.

tape was described (Frenk and Kocsis, 1974), but the component in the tape was not identified because the subject refused to be tested with the individual components.

Phenols are a common ingredient in germicidal disinfectants. A study describing five cases of depigmentation in one hospital and seven in another was reported (Kahn, 1970). The antiseptic used for cleaning surfaces in the hospital contained 4.1% of *o*-benzyl-*p*-chlorophenol (BCP) and 3% 4-*tert*-butylphenol (TBP). In addition, experimental studies were carried out on five volunteers who were tested with 6% TBP in 70% ethyl alcohol applied to the upper arm under occlusion. Maximal pigment loss occurred at approximately 1 month, and pigment returned in all subjects about 1 month later. Another group of subjects was tested with 6% hexachlorophene (HEX), *o*-phenyl phenol (OPP), BCP, and MBEH, and 1% solutions of *tert*-amyl phenol (TAP) and BCP. Depigmentation was produced in some of the subjects by all materials with the exception of MBEH. MBEH is capable of producing depigmentation as shown by other studies where a 20% solution was used (Lerner and Fitzpatrick, 1953; Becker and Spencer, 1962).

Exposure to depigmenting agents can and does occur if proper handling procedures are not practiced during the manufacture. Thirteen cases of leukoderma have been described among workers in a plant producing OPP and *p*-phenyl phenol (PPP) (Ito et al., 1968). Two cases of leukoderma in a

plant producing the monomethyl ether of hydroquinone or 4-hydroxyanisole (HA) were described, although 169 other men in the same plant were examined and showed no sign of depigmentation (O'Sullivan and Stevenson, 1981). HA is used as a stabilizer of vinylidene chloride, and two cases of leukoderma have been described in a plant where the material was being made (Chivers, 1972). A plant making TBP had 54 of 198 men with leukoderma; the intensity of the disorder was related to the degree of exposure (James et al., 1977). A total of nine cases of leukoderma was seen in two plants engaged in the production of TBP, butylated hydroxytolutene (BHT), and TBG (Romaguera and Grimalt, 1981).

In addition to the clinical observations, experimental studies have identified many compounds that cause hypomelanosis. Laundry ink containing *p*-cresol (CRE) produced depigmentation in CBA/J mice (Shelly, 1974). Thirty-three compounds were tested in black guinea pigs (Bleehen et al., 1968). Of these, 12 compounds produced depigmentation to some degree. Those that were very strong depigmenters were TBC, 4-isopropyl catechol (4IC), 4-methyl catechol (4MC), and catechol (CAT). Some produced definite but moderate hypopigmentation, among them, 3-isopropyl catechol (3IC), 3,5,-diisopropyl catechol (DIC), HY, 3-methyl catechol (3MC), and 3-methyl-5-*tert*-octyl catechol (MOC). Others produced definite but weak depigmentation: 3-*tert*-butyl 5-methyl catechol (BMC), 3,5-ditertiary butyl catechol (DTBC), and 4-*tert*-octyl catechol (4OC). Twenty-two additional compounds are listed in Table 24.1; some produced depigmentation and others did not. It is stated that substitution in the 4 position confers greater activity than the same substituent in the 3 position; for example, 4-methyl catechol is more potent than 3-methyl catechol. Some, but not all, compounds containing a sulfydryl group are capable of producing depigmentation. β-Mercaptoethylamine hydrochloride (MEA) and *N*-(2-mercaptoethyl)-dimethylamine hydrochloride (MEDA) were strong depigmenting agents. 3-Mercaptopropylamine hydrochloride and cystamine hydrochloride are weak to moderate depigmenters. Sulfanilic acid, cystamine, *bis*(2-amino-1-propyl)disulfide, 2-(*N,N*-dimethylamine)ethanethiol *S*-acetate, 2-mercaptopropylamine hydrochloride, and α-mercaptoacetamide were weak depigmenters. Another study compared HQ, MEA, and MEDA in black guinea pigs (Pathak et al., 1966). There is not as clear a pattern of structure–activity relationship among the thiols as there is with the phenols.

Another study on 23 compounds was carried out in black guinea pigs and black mice (Gellin et al., 1979). Strong depigmentation was found with HA, TBG, TAP, and MEBH. Moderate depigmentation was noted with HQ, TBP, phenol, and catechol. They failed to find depigmenting properties when testing butylated hydroxyanisole (BHA), BHT, octyl and propyl gallate, ethoxyquin gum guaiac, diethyl amine, hydrochloride, dilauryl thiodiproprionate, nonyl phenol, *o*-phenyl phenol, *p*-phenyl phenol, octyl phenol, nordihydroguaiarctic acid, and tocopherol. All the compounds mentioned last are used in a wide variety of products with which a large population comes in contact.

TABLE 24.1
Compounds Tested in Black Guinea Pigs

Compound	Depigmenting Potency
1,2,4-Trihydroxybenzene	0 to ±
2-Hydroxy-1,4-napthoquinone	0
2,3-Dihydroxybenzoic acid	0
Sulfanilic acid, pH 3.9	0
bis(2-Amino-1-propyl) disulfide	±
β-Mercaptoethylamine hydrochloride (MEA)	± to + +
2-Aminoethanethiol *S*-acetate	0
2-Mercaptoisopropyl amine	0 to ±
Hydrochloride x-Mercaptoacetic acid	0
2-Ethyl-*n*-hexyl-diphenylmethylene cyanoacetate	0
2,3,5,6-Tetrahydroxyquinone	0
2,3,5,6-Tetrahydroxyquinone	0
3,4-Dihydroxyphenylacetic acid	0
Sulfanilic acid, pH 7	0
bis(2-Aminoethyl) disulfide or cystamine	0 to ±
2-Hydroxypyridine	0
N-(2-mercaptoethyl) dimethylamine hydrochloride (MEDA)	± to + +
2-(*N,N*-dimethylamine) ethanethio-*S*-acetate	±
Cystamine hydrochloride	± to +
α-Mercaptoacetamide	0 to ±
1,3-Propane sultone	0
3-Hydroxypropane sodium sulfonate	0

Source: Bleehen, S.S., Pathak, M.A., Hori, Y., and Fitzpatrick, T.B., *J. Invest. Dermatol*., 50, 103–117, 1968.

Note: Criteria for assessing activity are as follows:

1. No visible depigmentation; skin color similar	0
2. Small spots or speckles of depigmentation	±
3. Uniform hypopigmentation	+
4. Complete depigmentation	+ +

24.4 REPIGMENTATION

Repigmentation after exposure to depigmenting agents is highly variable. Aside from individual variation, it is related to the degree and length of exposure to the agent. After application of MBEH for 30 days, repigmentation occurred 1 month after cessation of application (Denton et al., 1952). After workers ceased wearing rubber gloves containing MBEH, repigmentation commenced, but the degree of repigmentation is not stated (Oliver et al., 1939). Black subjects tested with 20 or 5% MBEH (Lerner and Fitzpatrick, 1953) had one subject who depigmented in 1 month and repigmentation was complete 2 months later. A case of depigmentation resulting from rubber swim goggles containing a depigmenting agent gradually repigmented over a 8 weeks period after use of the goggles was discontinued (Goette, 1984). MBEH was used to depigment black subjects, and in some, white patches remained after 2 years and the investigators speculated it might be permanent (Becker and Spencer, 1962). Some subjects tested with TBP repigmented within 6 months but others remained depigmented after 1 year (Kahn, 1970). Those areas that depigmented least, repigmented first.

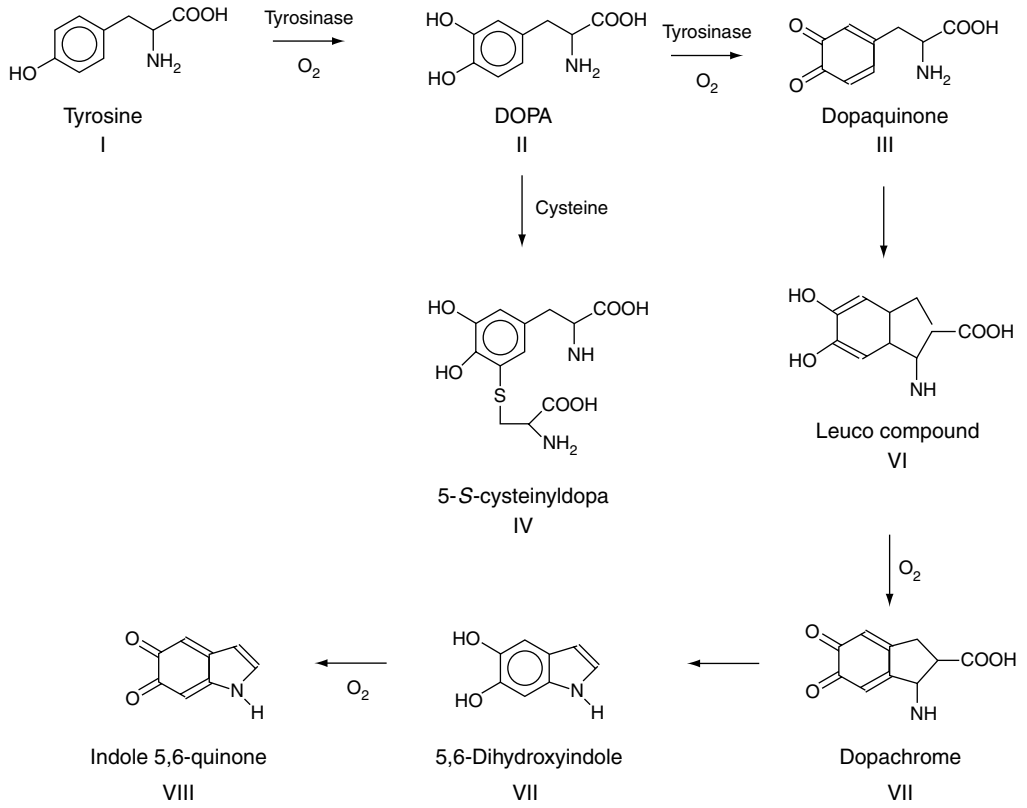


FIGURE 24.2 Steps in the synthesis of melanin (Lerner and Case, 1959). Indole 5,6-quinone undergoes condensation with proteins to form eumelanins, which are black or brown. The cysteine conjugate, 5-*S*-cysteinyldopa is further oxidized to a quinoid structure and then is conjugated with proteins to form pheomelanins, which are red or yellow in color. Tyrosinase activity can be diminished by substrate inhibition and, since several depigmenting agents with a phenolic structure can act as substrates, this may be one mechanism (Menter, 1988; McGuire and Hendee, 1971).

24.5 MECHANISM OF ACTION

The biosynthesis of melanin is a complex process that involves several steps, several of which are still not known. For example, several different protein structures can condense with indole quinone or 5-*S*-cysteinyldopa to give different colored pigments. Some of this is under genetic control and thus is different in individuals, as well as species. Some of this process is shown in Figure 24.2. Pigment formation can be disrupted by interference at any of these steps.

Seven mechanisms have been suggested by which the chemical agents could be producing depigmentation (Bleehen et al., 1968):

1. The agent may act selectively on a specific cell. The phenols do structurally resemble some of the intermediates involved in the synthesis of melanin, such as tyrosine or DOPA. Menter (1988) tested eight compounds as substrates for tyrosinase and found all of them to be suitable. Among them were the depigmenting agents TBC, 4MC, HA, and BHT. The presence of dopa-melanin enhances the action of tyrosinase on these substrates. It has been suggested that some of these products may act as antimetabolites and lead to degeneration or death of the cell (Lerner, 1971).
2. Chemical agents such as 4IC may react and tie up the active sites of tyrosinase. The molecule does not have the necessary side chain for conversion to the indole quinone and the reaction may bog down at step III in Figure 24.2.
3. The agent can inhibit the melanin formation by blocking the enzymatic oxidation of tyrosine to DOPA and the subsequent conversion to melanin.
4. The agent can interfere with the biosynthesis of the organelles—premelanosomes and melanosomes. Melanin is a free radical and produces a signal when analyzed by electron spin resonance. The addition of HA to the system changes and increases the signal (Riley, 1970). Free radicals are capable of generating peroxides and disrupting cell and organelle membranes by lipid peroxidation. Investigations by electron microscopy have shown disruption of melanosomes and destruction of membranous organelles in melanocytes (Jimbow et al., 1974).
5. The agent can interfere with the biosynthesis of the protein (e.g., by combining with the melanocytic ribosomes, which appear to be the sites for tyrosinase synthesis) (Jimbow et al., 1974). This may be another facet of lipid peroxidation.
6. The agent can interfere with the transfer of melanosomes to keratinocytes, either by inhibiting the

arborization of melanocytic dendrites or by causing intercellular edema. Irritation plays a role in pigment loss (Gellin et al., 1979; Becker and Spencer, 1962). Irritation is accompanied by edema, thus this may be a factor in depigmentation.

7. The agent can chemically alter the melanin present in the melanosomes. Because of the reducing action of some of the thiols, it appears that the dark-colored, oxidized form of melanin could be altered to the lighter-colored, reduced form. Since cysteine can condense with DOPA to yield pheomelanins, it may be possible for other thiols to be involved in a similar reaction.

It has also been suggested that depigmenting agents may act as an antigen after increased cellular permeability due to inflammation. An antibody is formed and this stops the formation of melanin granules. If antigen is produced in sufficient quantities, the reticuloendothelial system could respond (Becker and Spencer, 1962). This hypothesis has not been tested, but it may explain depigmentation at remote sites (Gellin et al., 1970).

Another mechanism that alters the level of glutathione reductase, which in turn affects the level of the reduced form of glutathione, may involve a change in the type of pigment produced (Yonemoto et al., 1983). Hairless mice treated with TBC showed an increase of the enzyme glutathione reductase. It was suggested that this change increases the level of reduced glutathione and in turn increases the number of pheomelanins, which are lighter in color than eumelanins.

Some important findings have been made using cultures of human melanoma cells (Del Marmol et al., 1993). In this study, intracellular glutathione was depleted by treating the cells with buthionine-*S*-sulfoximine (BSO). Tyrosine hydroxylase activity increased in parallel with glutathione depletion. The effect of thiols on melanogenesis can occur by at least two different mechanisms. First, low molecular weight thiol compounds can inhibit melanogenesis by direct interaction of the thiol groups with the tyrosinase active site, thus inhibiting tyrosine hydroxylation. Second, thiol groups are able to react with L-dopaquinone to form dopa-thiol conjugates that are pheomelanogenic precursors.

24.6 CONCLUSIONS

Many chemicals have been identified as depigmenting agents from clinical observations and experimental studies. These agents fall into primarily two categories: phenols and thiols. The most potent phenols are those containing an alkyl substitution in the 4 position. Those that are most irritating to the skin have the greatest potential for depigmentation. The mechanism by which depigmentation occurs is probably related to interference with one or more of the many steps of melanin biosynthesis. It is accompanied by destruction of melanocytes and their organelles. The structure–activity relationship of the thiols is much less defined. The mechanism of action of the thiols may be related to the depletion of glutathione or the involvement of the thiol in the place of glutathione in the formation of melanin. Fortunately, experimental methods have been developed to test compounds for depigmenting properties. Once the potential is recognized, proper protective measures can usually be instituted to minimize human exposure. The human is more sensitive than other species, and there is a large variation in sensitivity among individuals.

Recent literature reviews (PubMed and Embass) fail to reveal new depigmenting agents and mechanisms. We question whether this represents lack of interest, few reportable cases, or that the depigmenters have been identified and removed from the environment. The authors welcome new information that may have escaped our notice.

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25 Carcinogenesis: Current Trends in Skin Cancer Research

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25.1 INTRODUCTION

Skin cancer is the most prevalent malignancy in fair-skinned people (Diepgen and Mahler, 2002; Ley, 2002). The incidence of both the more common nonmelanoma skin cancer (NMSC) and the more lethal cutaneous melanomas continue to increase, reaching epidemic numbers. NMSC accounts for up to one-third of all cancers in the United States and Australia. According to recent population-based studies from Australia, the incidence rate is over 2% for basal cell carcinomas in males and 1% for squamous cell carcinomas. Mortality for NMSC is low. Surgical therapy is highly effective, but recurrence is frequent. Therefore, the associated morbidity is significant to the patient as is the burden on health care system. For melanoma, the American Cancer Society predicted there would be approximately 53,600 new cases in the United States during 2002, and 7400 deaths from melanoma during the same period. In Australia, there are over 50 new cases of melanoma per 100,000. The good news is that among cancers, skin cancer is believed to be one of the most preventable malignancies. Life style risks include ultraviolet radiation (UVR) exposure and diet. These factors can be modulated to decrease risk.

25.2 SUSCEPTIBILITY TO SKIN CANCER

Epidemiology studies and molecular data implicate exposure to UVR as the most important etiological factor for risk of skin cancer. While UVR plays a central role, other agents are clearly involved. These include the immune response, certain viral infections, genetic predisposition, and diet. As shown later, these risk factors are not exclusive.

25.2.1 Ultraviolet Light Radiation

UVR is the carcinogenic factor in sunlight. UVR has been mainly associated as a factor for NMSC, but some role for UVR has been suggested in malignant melanoma (Grossman and Leffell, 1997). Over time, accumulation of mutations induced by UVR contributes to cancer. Most mutations are repaired or damaged cells are removed by apoptosis. However, the additive effects of mutations involved in genes, involved in DNA repair, apoptosis, and control of the cell cycle can lead to tumor formation. UVR, like other agents that cause DNA damage, induces expression of the tumor suppressor gene p53, important for DNA repair, and apoptosis in response to DNA damage (Soehnge et al., 1997). It is, therefore, not surprising that many skin cancer cells have mutant p53. Mutations in p53 are present in about 56% of basal cell carcinomas (Lacour, 2002). Not only does UVR cause mutations in cellular DNA, but UVR also has profound effects on the cutaneous immune system. In an animal model of ultraviolet (UV) light-induced skin carcinogenesis, cyclooxygenase-2 was upregulated by UVR (Fischer, 2002), which would clearly stimulate inflammatory processes (An et al., 2002). Psoralen and ultraviolet A photochemotherapy (PUVA) is associated with a dose-dependent increase in the risk of nonmelanoma skin cancer (Studniberg and Weller, 1993). Like UVB radiation, PUVA is both mutagenic (Nataraj et al., 1997) and immunosuppressive (Ullrich, 1991) and therefore, can be considered a complete carcinogen.

25.2.2 Viruses

More and more evidence suggests that the human papillomaviruses (HPV) may have a role in promoting NMSC. These

viruses can affect tumor suppressors such as p53 and retinoblastoma (zur Hausen, 2000). Epidermodysplasia verruciformis, with a papillomavirus etiology, has long been regarded as a model for nonmelonotic skin cancer (Majewski and Jablonska, 2002). Flat warts occur in individuals with this disease, and cancers develop from lesions on sun-exposed areas. The presence (viral DNA) of a number of lesser-studied types of HPVs occur at a very high frequency in immunocompromised patients, for example, persons with renal transplants (de Villiers et al., 1997). These HPVs are also being found in lesions from immunocompetent patients as well (Biliris et al., 2000). Present data indicate that a primary infection occurs in the majority of individuals early in life. The virus remains latent, and prolonged UVR is needed to activate the virus or inactivate cellular genes responsible for controlled cell growth (de Villiers, 1998). Not only has UV light been shown to activate latent papillomaviruses (Amella et al., 1994), but also one of the viral proteins (E6) from diverse cutaneous HPV types inhibits apoptosis in response to UVR damage (Jackson and Storey, 2000). Studying HPV in skin cancer has been limited by detection methods. Currently, degenerate PCR methods are identifying many more HPV types and identifying HPV DNA in premalignant and malignant skin lesions (Biliris et al., 2000; Harwood et al., 1999). Consistent with the observations that HPV may be a cofactor for the development of skin cancers, several mouse strains exist with HPV transgenes and serve as models for progressive skin cancer (Arbeit et al., 1994; Kang et al., 2000). Other conditions add to the circumstantial evidence that HPVs are cofactors for skin cancer. The gradual loss of immunity in AIDS increases the cutaneous lesions associated with HPVs in these patients (Milburn et al., 1988), and increases in skin cancers are likely with long-term survivors of AIDS. An analysis of PUVA-related benign and malignant lesions found a prevalence of HPV DNA (Weinstock et al., 1995).

25.2.3 Genetic Disposition

The fair-skinned Caucasian population poses the greatest risk for skin cancer. Other genetic factors such as defects in DNA repair, the immune system, or detoxifying enzymes increase risk. For example, the transmission of Epidermo-dysplasia verruciformis (described earlier) is autosomal recessive, and the disease has the characteristic of decreased cell-mediated immunity (Cooper et al., 1990). Susceptibility loci have been identified (Ramoz et al., 2000). Individuals with functional polymorphisms in the detoxifying enzymes, glutathione-*S*-transferases (GSTM1 and GSTT1) show enhanced sensitivity to sunlight (Kerb et al., 2002), and such polymorphisms occur at high frequency among Caucasians. Moreover, these GST genotypes modulated Hypericum extract (St. John's wort)–induced photosensitization.

25.2.4 Immunity

Renal transplant patients have a well-documented risk of increased skin cancer. This increase is 50–100-fold (Birkeland et al., 1995). The cumulative incidence of skin cancer is 27–44% after 10–25 years of immunosuppression (London et al., 1995). This increase is concomitant with an increase in cutaneous lesions associated with HPVs; the virus is very often detected in both benign and malignant cutaneous lesions (Blessing et al., 1990; Benton et al., 1992).

25.2.5 Diet

A number of studies, which include animal and human studies, indicate that diet can affect skin cancer risk. A number of studies suggest that a high-fat diet may directly or indirectly affect skin cancer. For example, more skin cancers occurred at an accelerated rate in mice with transgenes for HPVs when the mice were given a diet with 20% corn oil (Qi et al., 2002). Grape skin and some other food products contain resveratrol, a phytoalexin. In a skin cancer model, it inhibited the development of preneoplastic lesions in a skin cancer model (Jang et al., 1997). This phytochemical is known to act as an antioxidant, induce phase II drug-metabolizing enzymes and inhibit cylooxygenase. Low selenium (a mineral found in garlic and related foods) levels in plasma have been linked to increasing risk of nonmelanoma skin cancer in humans. In a study of selenium deficiency using UVR-induced skin tumors in SKh:HR-hairless mice. Pence et al. (1994) showed the correlation between decreased risk with selenium deficiency, and that the deficiency resulted in decreases-glutathione peroxidase and increases in superoxide dismutase and catalase. In another study, men with basal cell and squamous cell cancers had lower levels of beta-carotine than controls. However, high intake of cruciferous vegetables, foods with beta-carotene and vitamin C, and fish were associated with a reduced risk (Lamberg, 1998). Support for antioxidants came from a cohort study in Great Britain showing a substantial protective effect for NMSC with vitamin E (Davies et al., 2002).

25.3 PREVENTION

Chemoprevention of photodamage and thus for photocarcinogenesis has been collectively referred to as photochemoprotection. Sunscreens and educational efforts may be an effective method to reduce UVR-induced photodamage. Additionally, many naturally occurring compounds such as antioxidants present in the diet have potential for human benefit. Fruits, vegetables, and certain beverages boost levels of antioxidants in the body and can serve as scavengers of sunlight-induced free radicals. For example, green tea, which is rich in polyphenolic antioxidants, has been suggested as an adjunct to sunscreen to prevent photodamage and subsequent skin cancer (Almad and Mukhtar, 2001).

A diet low in fat may reduce actinic keratosis and nonmelanoma skin cancers. Expanding intervention studies support that a diet with less than 20% of total calories is efficacious. Patients in low-fat groups had a significant lower rate of new cancers detected at 2 years (Jaax et al., 1997).

Consistent with animal studies, selenium appears to be beneficial. When a group of patients with a history of basal cell or squamous cell carcinomas were randomized into a

group of persons taking 200 mg selenium per day or placebo and followed for 6 years, the treatment showed a trend but not significance for reducing the incidence of skin cancers. However, the study was stopped because there was a significant reduction of total cancer mortality and total cancer incidence in the selenium group (Clark et al., 1996).

At a meeting of the American Academy of Dermatology (1998), Arbesman reported on the findings from more than 50 animal and human studies and suggested that the studies would support a diet of less than 20% calories from fat, five servings of fruit and vegetables, carotine from food equivalent to one and one-half medium carrots, vitamin E supplements, and selenium and vitamin C from food (Lamberg, 1998).

Together, studies (from basic science to intervention) support that life style change could dramatically reduce the incidence of skin cancers, particularly the NMSCs.

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26 Retinoids and Mechanisms of Their Toxicity

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26.1 INTRODUCTION

Retinoids comprise a large class of small lipophilic molecules related to vitamin A, retinol (ROL), in their chemical structure, biological activities, or receptor binding. The naturally occurring retinoids include ROL; retinyl esters (retinyl palmitate); 11-*cis*-retinal, 11cRAL; 9-*cis*-retinoic acid, 9cRA; all trans retinoic acid, tRA (tretinoin, Retin A); 13-*cis*-retinoic acid, 13cRA, (isotretinoin, Accutane); and as many as a few dozen active and inactive metabolites of these molecules. The provitamin beta-carotene may be included by extension as it can furnish retinyl esters when cleaved in the intestine. The synthetic retinoids now number well over 2000, only a few of which have been successfully commercialized as etretinate (Tigason, Tegison), acitretin (Soriatane, Neotigason), tazarotene (Tazorac), adapalene (Differin), and bexarotene (Targretin). This massive synthetic effort by a number of large pharmaceutical companies has been largely an attempt to obviate the toxicity of retinoids when administered in pharmacologic doses for treatment of various skin diseases and carcinomas but has had thus far limited success in divorcing desirable and undesirable biological actions.

Recognition of night blindness in ancient Egypt in the fifteenth century BC followed by recommendations in ancient Greece for its treatment by topically applied or ingested liver were proven to be prescient observations in the early twentieth century after discovery of the active principle vitamin A followed by its synthesis and animal and human experience involving dietary vitamin A for supplementation and pharmacologic therapy. The class is named after the retina, the site of the important interaction of 11-*cis*-retinal with opsin to form rhodopsin, the light sensitive pigment of the rods that is involved in night vision.

Both insufficient and excessive ingestion of vitamin A result in well-established clinical syndromes. Hypovitaminosis A manifesting as night blindness, keratomalacia, skin thickening and roughness, and embryologic defects is by far the most common presentation especially in developing countries where inadequate dietary vitamin A is a prevalent problem.[1] Hypervitaminosis A of the chronic type is more common than acute hypervitaminosis A and presents with dry, pruritic, erythematous, desquamating skin and lips, hair growth abnormalities, pain and tenderness of bones, anorexia, fatigue, irritability, headache, papilledema, and embryopathic manifestations in exposed fetuses as a result of overzealous dietary ingestion, which is more frequent, or intentional pharmacologic administration of vitamin A, which is now rather uncommon. Reports of acute

TABLE 26.1
Possible Factors in Retinoid Toxicity, General Aspects

Chemical structure, stereochemistry, chain isomerization, polarity, charge, lipophilicity
Absorption and first-pass metabolism
Plasma protein binding
Tissue distribution and localization within tissue
Pharmacokinetic parameters Cmax, Tmax, AUC, T1/2
Peak, trough and mean plasma levels of drug and its metabolites
Hepatic metabolism and activity and pharmacokinetics of metabolites
P450RAI (CYP26) induction, expression, and activity
Catabolism, glucuronidation, excretion, reabsorption
Possible competitive inhibition of enzymes
Specifics of placental transfer
Genetic polymorphisms and snps

TABLE 26.2
Possible Factors in Retinoid Toxicity, Specific Aspects

Local tissue-specific synthesis and metabolism
RAR α, β, γ isoforms and isotypes
RXR α, β, γ isoforms and isotypes
Receptor affinity and binding specifics
RAR/RXR dimerization
Heterodimerization of RXR with other nuclear receptors
Possibility and degree of upregulation of receptors by RA
RAREs of putative genes
Signal transduction pathways utilized or opposed
Transcription factors utilized or opposed
Hox-RARE interactions in embryogenesis
Other gene interactions
Regulatory feedback loops

hypervitaminosis A are rare as the retinoids generally have low toxicity in single doses even at substantial dose levels.[2] Few individuals have the opportunity to ingest large amounts of polar bear liver, which contains 12 mg retinol/gram and which in the past produced dramatic manifestations of acute hypervitaminosis A including headache, convulsions, coma, hallucinations, even death in early arctic explorers.

These observations and the long clinical experience of utilizing high-dose vitamin A for treatment of acne and disorders of keratinization that demonstrated substantial efficacy but also a side effect profile deemed undesirable, provided there was an stimulus for the synthetic retinoid programs. Thus the extremely enthusiastic reception of the marketing of Etretinate, Tigason in Europe for treatment of psoriasis and disorders of keratinization and isotretinoin, 13-*cis*-retinoic acid, Accutane in the United States in 1982 for treatment of the formerly nearly untreatable severe nodulo-cystic acne. In the United States, a storm of adverse events soon reported to the manufacturer, followed in 1983 by the first reports of human birth defects, tempered enthusiasm, and let loose an avalanche of governmental, regulatory agency, legal and congressional scrutiny. Presently these events have result in a highly restricted availability of isotretinoin and a marked dampening in the enthusiastic synthesis of retinoids, which once seemed such promising future therapy.

There has never been a time when it was more important to have a complete understanding of retinoid toxicity with a view to its abrogation or minimization. This chapter will address the issue of retinoid toxicity first in its broadest perspective (Table 26.1) and then in the specific details of its organ, cellular, and molecular origins (Table 26.2). The therapeutic effects of retinoids as well as the syndromes of hypo and hypervitaminosis A reveal several clues in many of their specific signs and symptoms of the critical and central role of vitamin A in development and maintenance of many, perhaps all organ and cellular systems. In the past three decades, a rapid accumulation of understanding of the basic mechanisms of biological actions of retinoids has resulted from the intense and now highly sophisticated research in chemistry, molecular and developmental biology, and genetics. This chapter will attempt to provide a sufficient and coherent background of present knowledge of retinoid action to allow a more complete understanding as the science continues to unfold. As much of the literature addresses the role of tRA and involves *in vitro*, animal *in vivo*, and transfection and transgenic experimentation, the observations cannot always be immediately extrapolated to effects in the human with therapeutic doses of tRA or other retinoids; but this molecule and the enormous literature documenting retinoid experimentation can serve as parts of a useful paradigm of action for the other retinoids. A working knowledge of the chemistry, pharmacokinetics, metabolism, and receptor binding of these natural and synthetic ligands is critical for an understanding of the complex mechanisms of their biological activities.

26.2 CHEMISTRY

Naturally occurring retinoids are small lipophilic molecules composed of a six-membered ionone ring that is essential to biological function, an unsaturated polyene side chain, which is unstable at its double bonds and subject to isomerization in several locations, and a terminal end group usually an alcohol, ester, aldehyde, or acid in structure (Figure 26.1). Synthetic retinoids have been classified as first-, second-, or third-generation compounds, based on the structural changes in the ring, side chain, and terminal group. The first generation is represented by the naturally occurring retinoids including 13cRA. In the second-generation retinoids, the ring is aromatized as with etretinate and acitretin and in the third generation, the so-called arotinoinds such as Ro 13-7410 and tazarotene, the side chain has been folded on itself to create one or more additional ring formations. Further modifications can produce molecules with retinoid activity but which ligand only RXR receptors. This is in contrast to most of the active first-, second-, and third-generation retinoids most of which are ligands of retinoic acid receptor (RAR) receptors, the

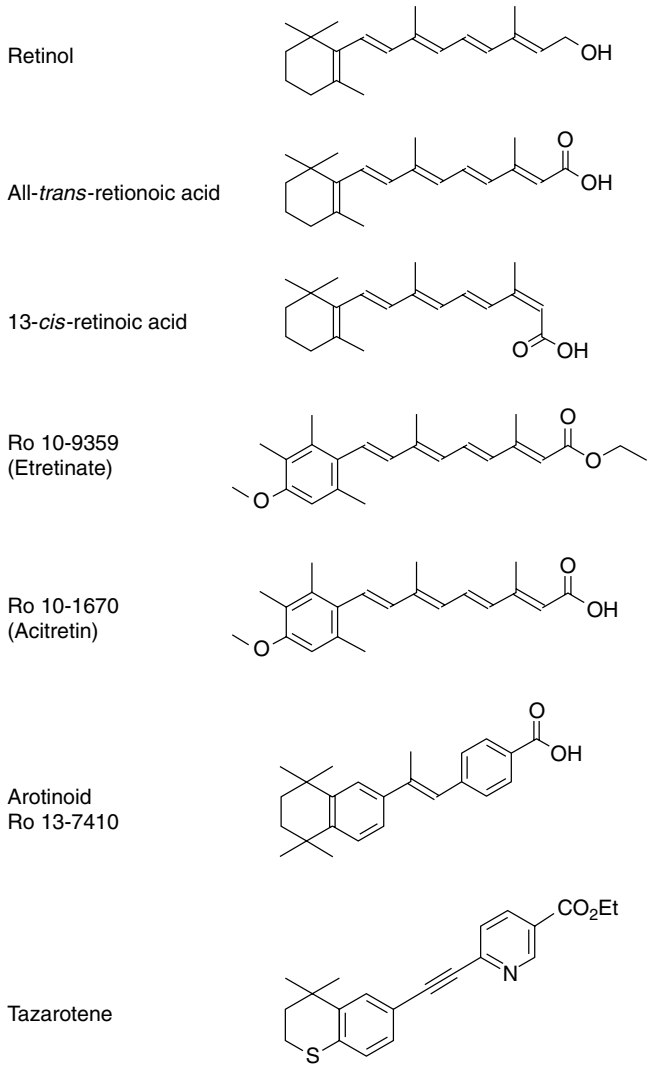


FIGURE 26.1 Structures of representative retinoids.

exception being 9cRA, which is also a ligand of RXR. These structural aspects are of considerable importance *in vivo* as they influence many of the critical aspects of absorption from the gut or skin, plasma protein binding and transport, pharmacokinetics, membrane transport—and as will be discussed later in some detail—enzyme interactions, and receptor binding.

Synthesis of several of the biologically natural retinoids can be accomplished *in vivo* via several enzyme systems provided that there is sufficient substrate of vitamin A equivalents available from dietary sources. Retinol and retinyl esters such as retinyl palmitate comprise the majority of dietary vitamin A equivalents with a smaller proportion obtained from carotenoids such as beta-carotene. Synthesis is generally in the direction of alcohol (retinol) to aldehyde (retinal) to acid (retinoic acid) through the action of a series of alcohol and aldehyde dehydrogenases. Thus the organism's requirement for both 11-*cis*-retinal for the visual cycle and tRA for most other functions is readily achieved with adequate retinol or retinyl ester ingestion.

26.3 ABSORPTION, TRANSPORT, AND TISSUE DISTRIBUTION

Most lipophilic drugs are absorbed only in the small intestine, and in their passage through the gut epithelium, they utilize membrane-bound protein receptors to facilitate their transport.[3] Dietary retinyl esters such as retinyl palmitate are cleaved to retinol in the small intestine by pancreatic enzymes and retinol is then absorbed into intestinal cells by a carrier-mediated process specifically utilizing cellular retinol binding protein 2, CRBPII. Retinol is re-esterified in the intestinal cell, incorporated into chylomicrons and reaches plasma via the lymphatics. Hepatocyte uptake by receptor-mediated internalization of chylomicrons allows hepatic storage or transfer of retinol to plasma bound to retinol-binding protein (RBP), a specific carrier protein synthesized and controlled by hepatic mechanisms. The importance of RBP is considerable in limiting the availability of free retinol to cells and RBP is one part of the complex and highly regulated system by which cellular retinol and thus retinoic acid are controlled. This system is lacking in administration of other retinoids and has potentially important consequences.

Most other retinoids such as tretinoin, isotretinoin, etretinate, and acitretin are poorly absorbed from the gut (e.g., approximately 60% for acitretin even with food) except in the presence of food, particularly high fat containing food where absorption can exceed 90%. Retinoids are transported nonspecifically mostly bound to plasma proteins, principally albumin, and thus their potential tissue availability is limited only by their plasma levels. Once in plasma, tissue distribution of retinoids is widespread but their specific local utilization and eventual biological activities and toxic consequences are highly complex processes, regulated by a number of factors, which will be subsequently elaborated.

26.4 PHARMACOKINETICS

A wide range in several pharmacokinetic parameters is encountered with therapeutic dosing of retinoids due to their inherently poor and variable bioavailability manifested by intrasubject variability and probable genetic differences manifesting as intersubject variability.

Mean plasma levels of endogenous retinoids are generally in the low single-digit or subnanogram range but can be elevated after high dietary intake of vitamin A equivalents or vitamin A containing food especially liver.[4] All therapeutic regimens with most retinoids produce plasma levels 10 to several hundred-fold than the endogenous levels.

All-trans retinoic acid is variably absorbed and its bioavailability is enhanced with concomitant food intake. Time to maximal plasma concentrations of tRA is 1–2 h and elimination is also rapid with a $T_{1/2}$ of 40–60 min. In several

studies of doses of 45–80 mg/m^2/day, AUC ranged from 387 μg/L/h to 682 μg/L/h.[5]

Isotretinoin is absorbed best when administered with food. A single 80 mg oral dose results in mean maximum blood concentrations of 256 ng/mL with a range of 167–459 ng/mL demonstrating high intersubject variability at a mean of 3.2 h with a similar variable range of 1–6 h.[6] These levels are 100–500-fold higher than mean endogenous levels of 13cRA. The major metabolite of 13cRA is 4-oxo-isotretinoin, where concentrations are 87–399 ng/mL at 6–20 h. Isomerization to the trans isomer also occurs resulting in significant levels of tRA. The parent compound (mean steady state 160 ng/mL) and metabolites are primarily bound to albumin in the circulation. The terminal elimination half-life of isotretinoin is 10–20 h and that of the 4-oxo metabolite is 17–50 h. Thus in spite of roughly equivalent plasma concentrations, there is a pronounced (up to 20-fold) difference in elimination of tRA or 13cRA. This results in a large pool of nonspecifically bound and potentially toxic 13cRA, tRA, and several of their active metabolites and isomers in plasma even at trough levels between daily doses.

Acitretin is also best absorbed in the presence of food, and after oral administration of a 50 mg dose it obtains maximal plasma concentrations in 2–5 h (mean 2.7 h) of 196–728 ng/mL (mean 416 ng/mL).[7] Metabolism and interconversion by isomerization of acitretin mainly to 13-*cis*-acitretin is followed by chain shortening and conjugations to water-soluble compounds eliminated via hepatic and renal excretion. The half-life of acitretin has been determined to be 33–96 h but for 13-*cis*-acitretin 35–148 hours. Furthermore, acitretin metabolism and elimination are significantly altered in the presence of alcohol that results in back conversion of acitretin to its parent acid form etretinate, a compound which has a very prolonged half-life of 84–168 days.

At least two conclusions can be drawn from these data: one, that the half-lives of these compounds demonstrate great disparity from a few hours to days to weeks and months; and second, that in the therapeutic dosing regimens usually employed, extremely high drug levels are maintained, which are up to several hundred-fold higher than usual endogenous and tightly controlled plasma levels. Thus these aspects may be considered to contribute to the overall toxicological profiles.

26.5 RETINOID METABOLISM

Metabolism generally, but not always, is a path to less therapeutically active and more water-soluble compounds that may be excreted in urine or bile. In the case of retinoids with side chains, changes in conformation due to isomerization may also occur spontaneously or as a result of enzymatic action, producing at times a somewhat confusing picture of still active metabolites and active stereoisomers. Studies of tRA, 13cRA, 9cRA, and their 4-oxo and other metabolites demonstrated that not only did many parent compounds and metabolites have substantial but relative binding affinities, but also that they were active in various cell culture assays with similar efficacy.[8] This has also recently been well demonstrated for

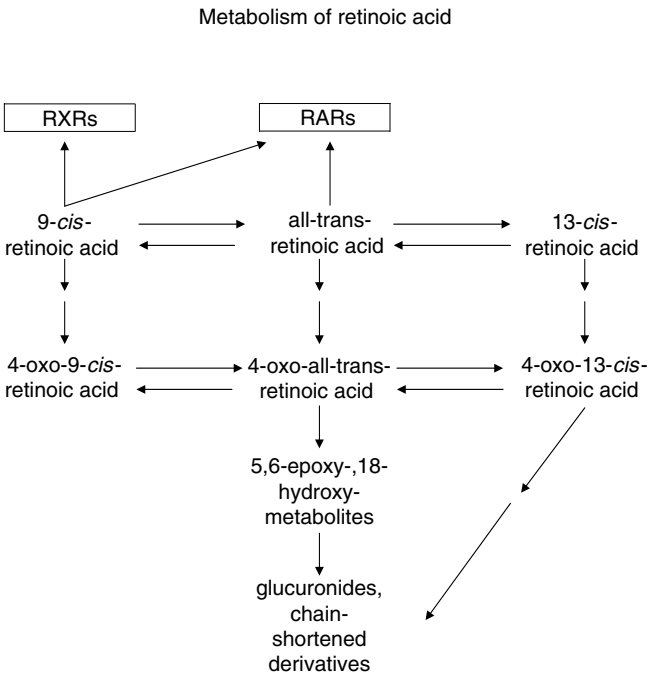


FIGURE 26.2 Metabolism of retinoic acid.

tRA, 13cRA, 9cRA, and their 4-oxo metabolites utilizing microarray and proteomic analyses that demonstrated substantial biological effect for the 4-oxo metabolites.[9]

Metabolism of the naturally occurring retinoids involves the successive hydroxylation, then oxidation of various parts of the molecule, followed by its glucuronidation to more water-soluble compounds that can be excreted in bile and urine (Figure 26.2). The principal enzymes of RA metabolism are cytochrome P450 enzymes *Cyp26A1*, *Cyp26B1*, and *Cyp26C1* that oxidize RA to more polar metabolites such as 4-oxo-RA, 4-OH-RA, and 18-OH-RA.[10] The gene for Cyp26A1 contains two functional retinoic acid receptor elements (RAREs) believed to contribute to its RA inducibilty, a factor particularly important in the developing embryo where RA synthesis and catabolism are tightly and reciprocally regulated. During midgestation *Cyp26A1* is expressed in the tailbud neuroepithelium and cervical mesenchyme; *Cyp26B1* is present in the hindbrain, branchial arches, and limb bud; and *Cyp26C1* is transiently expressed in the hindbrain.[11] There is a complimentary expression of these enzymes with retinaldehyde dehydroxigenase (RALDH) that is responsible for the locally restricted RA levels in the embryo.

26.6 BIOLOGICAL ACTIONS OF RETINOIDS

Naturally occurring retinoids are substrates for a number of enzymes, including those involved in their local synthesis for specific tissue needs and their local and systemic metabolic degradation. Enzyme interaction is particularly relevant in the eye's utilization of 11cRAL as a ligand of opsin to form rhodopsin, and where this synthesis of 11cRAL by

retinol dehydrogenase is a crucial step yet a possible site for interaction with other retinoids will be discussed later. The most interesting, relevant, and elucidated aspects of retinoid biology, however, involve retinoic acid's several interactions in the complex, redundant, highly regulated cellular control systems, particularly encompassing the signal transduction and nuclear transcription pathways of the cell.

Cellular homeostasis, function, and replication are regulated by integration of a combination of multiple influences including the cell's surrounding extracellular milieu, external signals such as growth factors, hormones, and cytokines, many mediated through signal transduction pathways, and of internal genetic pathways mediated by nuclear transcription. These pathways are complex, frequently highly redundant, tightly regulated, highly integrated, and reciprocally related and interactive with each other.

26.6.1 Retinoid Receptors and Gene Interactions

Retinoids act principally as ligands for two families of nuclear receptors, the (RAR), and its isotypes α, β, and γ and multiple isoforms and the retinoid X receptor, RXR, and its three isotypes α, β, and γ and their isoforms.[12] Retinoid receptors are members of the superfamily of nuclear receptors, which includes the receptors thyroid (TR), glucocorticoid (GR), androgen (AR), vitamin D (VDR), and peroxisome-proliferator-activated (PPAR) with which the RXR receptors can heterodimerize.

Nuclear receptors with their attached ligands function as transcription regulators of genes and bind to specific parts of the gene, a so-called response element usually of the promoter region (RARE). Transactivation of retinoid responsive genes is accomplished through an interaction of a heterodimer of RAR/RXR and its binding to the RARE, accompanied by ligand binding of tRA to the RAR ligand-binding domain (LBD), and an interaction of this complex bound to the RARE of the promoter with additional other so-called transcription factors, which include small proteins, phosphorylating kinases, and other molecules generated in the signal transduction pathway. Recent evidence has indicated that nuclear receptors may also bind in other ways to regions of genes and act with other transcription factors to transrepress, instances of which involve retinoids and will be discussed later.

Retinoid receptors demonstrate the same conserved modular structure as other nuclear receptors of the superfamily and similarly their amino acid sequences have been divided into regions A–F, each region being related to a different aspect of their functionality.[10] The central C region is the DNA binding domain (DBD) containing sequences of amino acids, which can bind to a RARE. The E region contains the LBD, as well as a transcriptional activation function, AF-2, and a dimerization surface for complexing with other nuclear receptors. The D region or hinge allows conformational change, a particularly important aspect of receptor function because of the exceedingly complex and conformationally restricted interactions of these various elements with the appropriate areas of the gene. The amino terminal end A/B regions contain a second transcriptional activation function termed AF-1.

RAREs are polymorphic, composed of hexameric motifs of the various combinations of nucleotides, and usually located in the regulatory area of the gene. The motifs are spaced most frequently by a 5 base pair (BP) or a 1 or 2 BP spacer. This polymorphism combined with the number of possible combinations of RAR and RXR isotopes and isoforms allows a large number of possible gene interactions and thus transactivation outcomes. The presence of a RARE does not always indicate RA responsiveness under physiologic conditions; however, a caveat, which is also very important, may apply to the many *in vitro* and gene transfection experiments where these genes may be vastly overexpressed.

The RARE is the primary site of interaction of the RAR/RXR heterodimer, which appears to be the preferred configuration of the two receptors and is the functional unit that transduces the retinoid signal *in vitro*. RAR or RXR can also form homodimers but these do not appear to be as important in most systems. RXR, however, can form heterodimers with large other nuclear receptors including GR, TR, VDR, PPAR, etc.; and these interactions may be important *in vivo*.

The most important natural ligand of RAR is tRA and this is overall the most important interaction physiologically. Although 9cRA also can bind RAR, a physiological importance has yet to be demonstrated for this ligand. Only 9cRA is a natural ligand of RXR but again, this binding has not been proven to be of significance *in vivo*. A very large number of other naturally occurring and synthetic retinoids can bind RAR with widely varying affinities, but the affinity of binding is not always related to the degree of its agonist or antagonist activities, the findings of which may be relevant to discussions of 13cRA. Interestingly, ligand binding of the RAR of the heterodimer RAR/RXR is determining for transactivation, but RXR need not always be liganded under physiological conditions and thus has been termed a transcriptionally active and silent partner in these circumstances. RXR heterodimerization with the TR, GR, etc. also follow this pattern of RXR being sometimes liganded and sometimes unliganded. In any case, the very presence of RXR confers specifics of conformation to all of these heterodimers and these allosteric effects may impact not only RARE binding but also binding of other transcription factors, which require their own space and positioning on the entire transcription complex.

In addition to the importance of the overall conformation of the RAR/RXR heterodimer in its binding to the RARE by its DBD and the possible conformational and other changes induced by liganding the LBD, the two activating function domains of the RAR or RXR also play an important role in the transcription machinery. AF-1 of the amino terminal A/B region is ligand-independent in its contribution but AF-2 is a receptor type-specific area of the LBD and thus is ligand specific. These two domains have varying amino acid sequences depending on the isotype of the receptor and act synergistically with each other and with other nuclear proteins called

transcriptional intermediary factors (TIFS), also called mediators or coactivators as part of the overall transcriptional machinery. There is evidence that autonomous transactivation that occurs in the absence of ligand could be related to an activating domain (AF-2 AD) in region E, the LBD.[10] Finally, the discovery of many putative coactivators that may react differently with various LBDs of the many isotopes and isoforms of the retinoid receptors further increases the possible combinations and permutations of diversity of transcriptional response to signals from retinoid liganding of receptors.

Transactivation does not appear to be the sole manifestation of retinoid signaling, however, as much evidence has been generated in the past decades indicating that retinoid signaling can also result in transrepression of genes. The mechanisms of transrepression have not been completely elucidated but it has been demonstrated that in transfected cells, RAR in the absence of its ligand can transrepress the basal level of promoter activity of target genes and that this is associated with an interaction of part of the LBD of RAR with a number of nuclear proteins that function as corepressors. Transrepression of the gene for AP-1, activating protein, by RAR was demonstrated in cultures of HeLa cells and to involve glycogen synthase kinase activation, negative regulation of junD hyperphosphorylation, and decreased RNA polymerase II recruitment. The investigators conclude that transrepressive effects of retinoids involve the RAR-dependent control of transcription factors and cofactor assembly on AP-1 regulated promoters.

As activation of the AP-1 complex is regulated through several of the mitogen-activated protein kinase (MAPK) signal transduction pathways described later, it is a clear demonstration of the cross talk or modulation now known to occur between signal transduction and nuclear signaling pathways. The complex structure of retinoid receptors and the marked allosteric changes induced in them by ligand binding help to explain the many possible interactions with the promoter of the gene and the many coactivators and corepressors of its transcription that are known to be functionally important.

Regulation of retinoid signaling is a complete feedback loop under precise systemic and local tissue control. RA induces its own receptor RAR and its own synthesizing RALDH and metabolizing CYP 450 enzymes. Finally, the ultimate regulation of retinoid signaling takes place after transcription when the ligand is released and the RAR is degraded via the ubiquitin/proteosome pathways. Agonistic ligands convert RARγ into a strong transcriptional activator. Concomitantly, RARγ is degraded by the ubiquitin-proteosome pathway.[13–17]

In 2002, over 532 genes had been proposed to have some degree of regulatory control by retinoids.[18] In this review of 1191 reports in the literature, 27 genes were classified as direct targets of retinoid control and another 105 genes were candidates pending additional confirmatory investigation. In a recent study of acne lesions, 211 genes were found to be upregulated, including those known to be associated with inflammation and scarring and several known to be retinoid sensitive.[19] These studies indicate the likelihood of an overall importance of these ligands in gene regulation of biological processes. In the sections that follow, the specific and critical nature of a few of these genes involved in embryologic development and epidermal differentiation will be presented. It is intuitive that perturbation of these pathways by administration of excessive natural or synthetic ligands may have significant consequences and manifest as toxicity.

26.6.2 Cell Signaling–Signal Transduction

The integration of internal and external cellular events is mediated primarily through a large number of transmembrane proteins serving in some cases as regulated conduits as in calcium and sodium channels, as facilitators of drug or molecule transport through the plasma membrane as the g-protein coupled receptors (GPCR), and as signaling moieties as in membrane receptors of signal transduction that are widely utilized by the immune and other cellular control systems.

Signal transduction is essentially a system of pathways by which an external stimulus to a cell is received, interpreted, or translated into a message by a membrane protein, then transduced through multiple second messengers in cytoplasmic pathways involving phosphorylations and dephosphorylations by protein kinases and phosphatases, and ultimately, by generation of a number of different transcriptional mediators that interact with nuclear receptors to transactivate appropriate genes. The stimuli are as diverse as exposure to light, (as in the g-protein coupled receptor, GPCR, signaling of rhodopsin), exposure to growth factors, hormones and drugs, or events of immune system functioning. The mechanisms by which the translation and transduction occur are frequently interactive (cross talk), redundant, utilized by diverse stimuli, and reciprocal with nuclear signaling pathways.

The signal transduction pathway is too complex to describe here in detail but a conceptual overview applicable in general to cell signaling will be helpful in understanding the complexities of retinoid action. Membrane receptors are generally complex proteins composed of an extracellular domain that is an LBD, a transmembrane domain sometimes of considerable complexity, and a cytoplasmic domain that activates protein tyrosine kinases that are either an intrinsic part of its cytoplasmic domain and that can self-phosphorylate or that bind with and are phosphorylated by cytoplasmic receptor–associated tyrosine kinases. Phosphorylation has several important aspects: it results in activation of the molecule, particularly important for enzymes; it results in conformational change that allows recruitment and attachment of other signaling proteins; it is quickly accomplished enzymatically through high-energy adenosine triphosphate (ATP) or guanosine triphosphate (GTP); and it is rapidly reversible. The initial signaling protein is frequently the enzyme phospholipase C-gamma (PLC-gamma), which after phosphorylation in this membrane process is activated and cleaves the membrane phospholipid phosphatidylinositol biphosphate (PIP_2) into two parts, inositol triphosphate (IP_3) and diacylglycerol (DAG). IP_3 acts with its receptor in the endoplasmic reticulum to cause the release of Ca^{2+} into the cytosol elevating intracellular free Ca^{2+} several fold, and

triggering the opening of Ca channels allowing more Ca into the cell and sustaining the signal. DAG also participates in the activation of members of the PKC family. Finally, as part of this initial signal generation, adaptor proteins that mediate protein–protein interactions through their SH2 and SH3 domains facilitate binding of phosphotyrosine residues and proline-rich regions in this initial signaling complex of multiple proteins and other molecules.

Propagation of the signal thus generated from the tyrosine kinase–associated receptor next activates a class of small GTP-binding proteins (small G proteins), the best-known example of which is the *Ras* oncogene protein product Ras. The small G proteins exist as inactive GDP-bound forms and active GTP-bound forms the activation being mediated by guanine-nucleotide exchange factors (GEFs) that exchange GDP for GTP. The active G-proteins, as a source of high-energy phosphate through their GTP, participate in the activation of the subsequent cascade of successive phosphorylations of the MAPK cascade that result in phosphorylation and activation of transcription factors such as AP1 in the nucleus. MAPK consists of at least 10 different families, 2 of which, extracellular signal-related kinases 1 and 2 (ERK1 and -2), are activated through growth factor receptors and by phorbol esters such as TPA, the overall pathway being designated as $MAPK^{ERK}$. A related module is termed $MAPK^{JNK}$ where the kinase is a jun *N*-terminal kinase. This pathway is activated by many stress stimuli including UV (ultraviolet) light, osmotic stress, and proinflammatory cytokines such as TNF and IL-1. Stressful stimuli and proinflammatory cytokines can also active the $MAPK^{p38}$ pathway.

A classic example of transcription factors, the AP-1 family is composed of various combinations of jun (c-jun, junB, and junD) and fos (c-fos, fosB, fra1, and fra2) proteins that are themselves products of the proto-oncogenes *jun* and *fos*. These transcription factors are important participants in the nuclear events that result in gene transactivation and transrepression. Retinoid signaling may be involved in both of these processes.

Many details of this generic signal transduction pathway have been omitted in this brief overview and many important variants of signal transduction pathways have not been discussed but most pathways follow this general paradigm. As some of these additional components are relevant to retinoid activity they will be discussed specifically in that context.

Some signal transduction pathways are enhanced by retinoids, an example being induction of IL-8 production accompanied by enhanced IL-8 mRNA expression in cultured human keratinocytes treated with 10^{-7} M tRA.[20] Increased expression of NFkB was accompanied by increased expression levels of its constituents p65, RelB, p52, and p50, increased DNA-binding of p65, and phosphoylation of IkB, the inhibitor of NFkB activation. As the IL-8 gene does not contain a classical RARE, it was concluded that the action of this retinoid is mediated through these secondary events described rather than through transactivation. IL-8 is an inflammatory cytokine that could play a role in the skin irritation observed during topical retinoid treatment.

A number of signaling pathways are operative in keratinocytes including different receptors and signaling pathways of signal transduction utilized by IL-1, TNF-α, or TGF-α, which while varying in their specifics of receptor type; specific kinases employed; and signaling proteins involved; all achieve in the end, one or more transcription factors such as AP-1 and NFkB.[21]

In some circumstances, retinoids oppose the direction of signal transduction, a good example being the frequently reported inhibition of AP-1 activation. This opposition to the direction of signal transduction may also be important to keep in mind when interpreting *in vitro* results where signal transduction is lacking and retinoid signaling is therefore unopposed.[22] These aspects will be further discussed in relation to effects of retinoids on skin.

Inhibition of the metaloproteinases (MMPs), collagenase, gelatinase, and stromelysin has been demonstrated after pretreatment of skin with topical tRA prior to UV irradiation and is mediated by repression of AP-1 activity through inhibition of its c-jun component.[23,24]

26.6.3 Apoptosis

Apoptosis is a pathway of cell death that is induced by a tightly regulated intracellular program in which cells destined to die activate enzymes (including caspases) that degrade the cell's own nuclear DNA and cytoplasmic proteins.[25] Although its structure is sufficiently altered to make it a target of phagocytosis, the cell's plasma membrane remains intact without sufficient leakage of cellular contents to provoke inflammation. The process occurs in skin, the "sunburn cell" of the epidermis being well described as an apoptotic cell resulting from irreparable UV damage, but it occurs in many other circumstances and organs. Retinoids can induce apoptosis in many *in vivo* and *in vitro* circumstances including keratinocyte cultures and skin equivalents.[26,27] In keratinocyte cultures and skin equivalents exposed to UV, tRA strongly increased the mRNA and protein expression of p53 and caspase-3, -6, -7, and -9, which are important regulators of apoptosis.[28] In cell cultures of CTCL lines MJ, Hut78, and HH, bexarotene induced apoptosis in a dose-dependant manner as demonstrated by activation of caspase-3, decreased levels of survivin, and cleavage of poly(ADP-ribose) polymerase without effect on expression of Fas/Fas ligand.[29]

26.7 RETINOIDS IN SKIN

There has been extensive research in the past few decades investigating the specific outcomes and mechanisms of retinoid effects. Skin biology has progressed in that same time frame and revealed a complexity that has been exhilarating yet at times frustrating when specific mechanisms of action such as that of retinoids were sought. One of the most confusing aspects of retinoid biology was the frequent and very differing results of *in vitro* and *in vivo* investigation, particularly when tumor cell lines or immortalized lines were studied or even normal human keratinocytes were cultured under

different conditions or exposed to high or low concentrations of retinoids.

Retinoids are normally involved in several regulatory pathways that involve the homeostasis, growth, and differentiation of keratinocytes and some functions of fibroblasts. As in other systems, most if not all of the actions of retinoids in the skin can be traced to their functions as ligands for their retinoid receptors. The principal retinoid receptors in the skin are RARγ and RXRα, which, as elsewhere, form heterodimers in their binding to RARE. The recent study of acne cited earlier revealed 211 genes that were upregulated in acne lesions, many of which are known to be regulated or influenced by retinoids. This gives one an idea of the potential scope of retinoid action in the skin and in many of these instances, RARE have been identified and the gene sequenced, manipulated by transfection, or mutated and knocked out in mice, providing an enormous database to be digested and understood.

In vivo, retinoids primarily produce epithelial hyperproliferation that can be demonstrated histologically by a thickened epidermis, acanthosis, more space surrounding keratinocytes and fewer desmosomes, hypergranulosis, and a thinner and more compact stratum corneum.[30] These aspects are mirrored ultrastructurally by fewer desmosomes, decreased tonofilaments, enhanced keratinocyte autolysis, and intercellular deposition of glycoconjugates that one group has demonstrated to be hyaluronate.[31–33] Clinically the skin manifests erythema; scaling; a loosened stratum corneum; a tendency for dysadhesion; and by instrumental techniques, increased transepidermal water loss.[34]

Numerous investigations utilizing human epidermal cells or neoplastic epidermal cells in culture have demonstrated that multiple markers of epidermal differentiation such as keratin, involucrin, and loricrin, expression are altered and have concluded that retinoids primarily effect differentiation. In human skin *in vivo*, however, it has been repeatedly demonstrated that the primary effect of retinoid treatment is hyperproliferation. Chronic application of topical tRA to photodamaged human skin demonstrated epidermal thickening (25 of 25 samples), stratum granulosum thickening (15 of 25), parakeratosis (13 of 25), a marked increase in the number of cell layers expressing transglutaminase (13 of 25), and focal expression of keratin 6 (12 of 25), and keratin 13 (8 of 25). Three major differentiation products keratins K1, K10, and K14 were not altered.[35] Essential confirmation of these results in biopsies of buttock skin of normal volunteers treated with topical tRA again demonstrated that hyperproliferation is the primary effect of topical retinoid treatment and that markers of differentiation such as keratin, involucrin, and filaggrin do not substantiate primary differentiation effects.[36]

Proliferation of skin can be caused by many external stimuli but while the specific mechanisms may vary, it appears that signal transduction pathways are involved in most instances. Epidermal proliferation as a result of injury or stress may involve cytokine stimuli such as IL-1 or TNF-α, each acting via differing yet cross-modulating signal transduction pathways. One of the most important and common initiators of epidermal proliferation is epidermal growth factor (EGF), an autocrine and paracrine ligand for the family of keratinocyte receptors EGFR. This important mitogenic pathway can be initiated by a number of related growth factors including the ligands EGF; TGF-α; amphiregulin (AR); and heparin-binding EGF (HB-EGF). This is a typical signal transduction pathway involving ras as an intermediate protein and ERKs as kinases.

Very convincing evidence has recently been put forth demonstrating that the proliferation of skin observed with retinoid treatment is related to the induction of ligands HB-EGF and AR and their subsequent effects on keratinocyte proliferation via this signal transduction pathway.[34,37] Demonstration that mRNA levels for HB-EGF and AR were several-fold increased in skin treated with topical tRA was confirmed by increased protein levels of AR in human skin and increased processed forms of AR and HB-EGF proteins in skin organ cultures (the investigators were unable to reliably extract HB-EGF from skin samples). Confirmation that signal transduction is involved was demonstrated by a 2.5-fold increase in ERK1/2 phosphorylation compared to vehicle. Blocking EGFR kinase activity by the inhibitor genistein reduced epidermal cell growth. These results are very convincing of a retinoid-EGFR signal transduction pathway link but do not further elucidate the mechanism of action of the retinoid. As no putative RA response elements have yet been demonstrated for the gene for HB-EGF, alternate mechanisms than gene transactivation may be operative.[38] Some of the observations of putative retinoid action in signal transduction pathways discussed earlier may be relevant.

Profound suppression of sebum has been presumed to be one of the principle actions by which 13cRA exerts its therapeutic effect on acne but the mechanism of sebum suppression remained undetermined. Utilizing immortalized SEB-1 sebocytes, recent work has demonstrated that 13cRA but not 9cRA or tRA inhibits growth and induces apoptosis, and these effects are accompanied by decreased DNA synthesis, an increase by 2.64-fold in the expression of the cell cycle inhibitor p21, and a 3.58-fold increase in cleaved caspase3 in sebocyctes.[39]

The mechanism of hair loss during retinoid therapy had likewise remained unclear but a recent report may provide some clarity. Human scalp hair follicles (HF) grown in culture and treated with 10^{-8} or 10^{-10} M tRA demonstrated that at day six, 80% had prematurely entered catagen compared to the control group.[40] An upregulation of apoptotic and a downregulation of Ki67 positive cells were noted in tRA-treated HF. Significant upregulation of TGF-β2 was observed in anagen HF in the dermal papilla and the dermal sheath. As TGF-β2 has demonstrated capacity to stimulate keratinocyte apoptosis, it may contribute to the mechanism of hair loss.[41]

26.8 RETINOIDS IN THE VISUAL SYSTEM

Normal vision is highly dependant on the availability and conversion of retinol to 11-cis-retinal, 11cRAL and the interaction of 11cRAL with opsin to form the light-sensitive GPCP,

g protein coupled receptor rhodopsin utilized particularly by the rods of the retina in low light conditions.[42] Only retinol and not retinoic acid can be converted to 11cRAL in this system and a deficiency of retinol results in impairment of night vision of varying severity depending on degree and duration of deficiency. Dietary tROL is isomerized in the eye to 11cROL that in turn is converted to 11cRAL by dehydrogenase enzymes.

Exposure of rhodopsin to light results in isomerization of the chromophore 11cRAL to 11tRAL and generation of an action potential-like signal (phototransduction) that is amplified and propagated in the retina, conveyed via the optic nerve and interpreted by the brain as vision. After the dissociation of 11tRAL it is isomerized back to 11cRAL, which is then again available as a ligand for opsin to again generate rhodopsin. As these reactions occur very rapidly in microseconds to seconds, a sufficient source of 11cRAL must always be available to enable rhodopsin formation and thus normal rod vision in low light conditions.

A number of retinoids including 13cRA have been reported to be associated with decrease in night vision. Night vision decrease in three of 50 patients receiving 13cRA at a dose of 1 mg/kg/day was accompanied by abnormal dark adaptation curves in two patients, abnormal ERKs in two patients and a mildly abnormal electrooculogram in one patient.[43]

It has been demonstrated that tRA and 13cRA inhibit ocular retinol dehydrogenases *in vitro* in an amphibian system hindering formation of 11cRAL from its 11cROL precursor.[44] Inhibition of the isomerases involved in the isomerization of all-trans retinal to 11-*cis*-retinal was not, however, observed in this system. No impairment of rod function was demonstrable by electroretinography, ERK in rats treated with 13cRA but regeneration of rhodopsin was slowed 50-fold. The experimental findings were consistent with 13cRA inhibition of the retinoid isomerase and of the 11-*cis*-retinol dehydrogenase.[45] The experimental conditions did not allow a determination of whether competitive enzyme inhibition or other mechanisms were operative however.

Photoreceptor-specific retinol dehydrogenase (prRDH) catalyses reduction of all-trans-retinal to all-trans-retinol and prRDH knock-out mice ($prRDH^{-/-}$) demonstrated delayed recovery of rod function on ERK demonstrating the importance of this enzyme system for rod recovery.[46] Demonstration *in vitro* and *in vivo* of the capacity of positively charged retinoids to inhibit isomerization in the retinoid cycle of retinal pigmented epithelium was further refined in additional work by the same group that indicated competitive inhibition of lecithin:retinol acyltransferase (LRAT), an enzyme that catalyzes the transfer of an acyl group from phosphatidylcholine to all-trans-retinol, thus interfering with an additional step in the retinoid cycle that must quickly regenerate 11cRAL, the ligand for opsin.[47,48]

Taken together, the evidence suggests retinoid interference in rod recovery, by their possible inhibition of a number of enzymatic steps in the generation of 11cRAL. Further evidence of this type of inhibition in other systems is that tRA and a number of synthetic retinoids have been demonstrated to be classical competitive enzyme inhibitors for the enzyme ribonuclease P (RNase P) in keratinocytes.[49]

26.9 RETINOIDS IN LIPID METABOLISM

Dyslipidemias are frequently observed during retinoid therapy. Although the true toxicity of dyslipidemias is not the elevation or decrease of specific lipids *per se*, but rather the long-term consequences of the perturbation measured primarily by cardiovascular pathology, in no other of the retinoid toxicities is the undesirable effect as easily quantitated in frequency and magnitude as in that of abnormalities of lipid metabolism. Furthermore, there is in these a single end point easily measured by a simple blood test, a large clinical population already studied for the perturbation and to some extent the long-term effect, and importantly a significant degree of understanding of the cellular and subcellular initiating activities. This may be the optimal paradigm for retinoid toxicity with potential application to the understanding of other retinoid toxic manifestations.

Dietary lipids and those synthesized in the liver (carrier proteins, enzymes, and apoproteins) interact in a highly complex system, which is substantially perturbed by therapeutic levels of retinoids. Dyslipidemia involving serum triglyceride elevations of over 800 mg/dL (as chylomicrons and very low density lipoprotein, (VLDL)), cholesterol elevations (as low density lipoprotein, (LDL)), and high density lipoprotein (HDL) decreases occurred in approximately 25, 7, and 15%, respectively, among young individuals treated for severe nodular acne with isotretinoin in standard therapeutic doses of 1 mg/kg/day.[50] Very similar incidences and severities are observed with etretinate or acitretin therapy in spite of the pronounced differences in structure, absorption, distribution, metabolism, and excretion (ADME), disease indication, and age and health of the treated populations.

Dietary lipids and endogenous lipids synthesized by the liver are carried in the blood and delivered to sites of utilization packaged in spherical structures (chylomicrons, VLDL, LDL, HDL) containing various combinations of triglycerides (TG), cholesterol, cholesterol esters, and phospholipids.[51] These structures also incorporate, as an essential component, one or more hepatic-synthesized apolipoproteins that function in various ways as structural components, ligands, cofactors for enzymatic reactions, or activators or inactivators of lipoprotein lipase (LPL).

26.9.1 TRIGLYCERIDES

Several enzymes are involved in lipid metabolism notably LPL, the major plasma lipolytic enzyme, which hydrolyses TG from chylomicrons and VLDL. Apolipoprotein C-III, apo C-III has several functions including the inhibition of LPL and possibly also inhibition of hepatic uptake of chylomicron and VLDL remnants. Most studies in humans do not demonstrate retinoid effects on LPL but substantial evidence indicates that retinoids may induce apo C-III gene expression. Increase in apo C-III also appears to inhibit the binding of TG-rich particles to endothelial surfaces. Thus direct effects

on the major catabolic enzyme of TG as well as inhibition of their uptake and thus utilization by target cells or metabolism by liver would contribute to the cause of elevations of TG.

Induction of apo C-III gene expression has been demonstrated in human hepatocytes and other cell systems and specificity for RXR agonists is demonstrated. Gene transcription of apo C-III is by a DR-1-like response element. It is further noted that several other nuclear receptors, such as TR and PPAR, may also have a role in regulation of the apo C-III gene at the same site, perhaps mediated by heterodimerization with RXR.

26.9.2 HDL

HDL attracts and carries cholesterol from cell membranes and delivers it for hepatic metabolism and excretion into the bile. HDL incorporate many apolipoproteins but apo A-1 is particularly important in its role in "reverse cholesterol transport," particularly, for example, in cholesterol removal from atherosclerotic lesion macrophages.

Although studies in many cell lines demonstrate a retinoid effect via receptor binding to the gene for apo A-I and apo A-II, in humans the levels of these apolipoproteins are not affected. Retinoids have demonstrated binding and activation at the specific response element for cholesteryl ester transfer protein (CETP) transcription. This plasma protein is involved in the exchange of cholesteryl esters for TG from HDL to VLDL and LDL. The explanation for decreases in HDL with retinoid therapy requires additional information but likely involves retinoid effects on specific gene response elements affecting the lipids, enzymes, or apolipoproteins involved with HDL.

26.10 RETINOIDS IN EMBRYOLOGIC DEVELOPMENT

Normal embryologic development is an extremely complicated and exquisitely regulated process in which retinoids, specifically tRA, play a critical role. Perturbation of this process by deficiency or excess tRA or excess agonistic retinoid or the wrong timing or placement of tRA's presence locally can lead to significant embryotoxicity ranging from minor developmental defects through major malformations, to embryolethality depending on the degree and timing of the perturbation. The active ligand in normal embryologic development is tRA whose local synthesis, metabolism, nuclear receptor, and gene transactivation are tightly regulated and which factors in turn determine the dose, timing, and the specific localization of ligand available for receptor binding and transactivation of appropriate genes.

Nearly all that is detailed in the preceding text regarding synthesis, metabolism, pharmacokinetics, receptor-binding aspects, and signal transduction apply in some way to embryotoxicity and must be borne in mind in the background of the very specific events that take place during embryogenesis and that have been elaborated in some detail in the past two decades utilizing a highly sophisticated array of biochemical and genetic techniques.

Embryogenesis, by the very nature of its dynamic events from mitosis to organ development and anterior–posterior (AP), cephalo–caudal, and side-to-side orientation and patterning, must be strictly regulated by transactivation and transrepression of multiple interactive genes and by strict regulation of signal transduction and enzymatic pathways. Retinoic acid (RA), sonic hedgehog (Shh), and fibroblast growth factor (fbg) pathways appear to play important signaling roles in conjunction with the *Hox* genes of the Homeobox family, and with *Raldh* and *Cdx* genes. At least 39 murine hox genes in four clusters *Hoxa* to *Hoxd* have been described and four *Raldh* 1–4, and four *Cdx* 1–4. Other genes are undoubtedly involved but have not been as well characterized.

A number of experiments utilizing dietary manipulation and gene knock-out mutants such as *Raldh2*$^{-/-}$ have elucidated the role of tRA in early development. Maternally supplied retinol is converted to tRA in the developing embryo. tRA is synthesized in the embryonic paraxial mesoderm by retinal dehydrogenase 2 (Raldh2/Aldh1a2) and induces *Hox* expression in the adjacent neural tube. RA concentration is spatiotemporally regulated by this local synthesis, and by local inactivation by cytochrome P450 enzymes. Disruption of the murine *Raldh2* gene precludes tRA synthesis and results in midgestational lethality accompanied by impairment of morphological segmentation throughout the hindbrain of *Raldh2*$^{-/-}$ embryos.[52] Additional RA-generating activities in the differentiating forebrain, hindbrain, and spinal cord are attributable to RALDH1 and RALDH3 enzymes, which may be operative in experiments of embryonic rescue from lethality by maternal administration of RA.[53]

RA-supplemented *Raldh2*$^{-/-}$ embryos exhibit impaired development of their posterior (third–sixth) branchial arch region and this affects the development of its derivatives including aortic arches, pouch-derived organs (thymus, parathyroid glands), and postotic neural crest cells that adversely affects the posterior (9th–12th) cranial nerves.[54] The embryonic heart tube of these animals forms properly but fails to undergo rightward looping and instead forms a medial distended cavity.[55] Embryonic RA synthesis is thus required for realization of heart looping, development of posterior chambers, and proper differentiation of ventricular cardiomyocytes. In mice, *Raldh3* knockout suppresses RA synthesis and causes malformations restricted to ocular and nasal regions.[56] The accumulated evidence thus suggests that there is some specificity as well as some overlap of functions of the various RALDH enzymes.

At, or shortly following, gastrulation, the unsegmented paraxial mesoderm begins to regionalize into segments that will eventually result in anterior–posterior patterning of neural, head and neck, vertebral, and cardiac structures. In the mouse, *Hox* expression is initiated at embryonic day

7.5 (E7.5) in the primitive streak and this expression is aligned in an AP direction in spatially restricted expression domains, sometimes termed a Hox code.[57] This spatiotemporal code is important in determining and enabling AP structural development as seen in the hindbrain, cranial nerves, vertebrae, heart, etc. An example relevant to development of all these structures is the observation that at least 11 Hox genes from paralogue groups 1–4 must be properly aligned at specific interfaces between rhombomeres to correctly pattern the normal repertoire of cell fates along the AP axis of the developing hindbrain.[58] *Rarb* is a direct transcriptional target of *Hoxb4*, thus this study identifies a new molecular link completing a feedback circuit between *Rarb*, *Hoxb4,* and *Hoxd4*. The timing of *Raldh2* expression and thus tRA synthesis correlates with Hox-inducing activity, and the segmental expression of this gene family is part of the process by which AP positional identity and final cell fate is determined. Although initiation of Hox gene expression is highly dependent on the signal from tRA, other transcription factors are likely to be involved in transactivation and all of these components must be present at the appropriate location, level, and timing for the correct outcome. RAREs have been characterized for *Hoxa1*, *Hoxb1*, *Hoxb4*, and *Hoxd4* that bind RAR/RXR heterodimers. Through a combination of biochemical, genetic, and transgenic techniques, it has been determined first, that *Rarb* uses a two-step transcriptional mechanism: induction by a mesodermal RA signal and maintenance by *Hoxb4* and *Hoxd4*; and second, that *Rarb* is a direct transcriptional target of *Hoxb4*. Transcriptional regulation between the Hox and RAR gene families is thus bidirectional. Furthermore, it was demonstrated that RA produced by the mesoderm is required to induce the early neural expression of *Rarb*. A complex genetic circuit has thus been described that involves direct and indirect interactions among *Rarb*, *Hoxb4*, and *Hoxd4* and that forms a transcriptional feedback circuit to maintain gene expression and to align the expression borders of multiple Hox and RAR genes at a segment boundary.

The Cdx family of genes has also been implicated in embryologic development and as regulators of Hox gene expression due to the evidence that murine *Cdx1*, *Cdx2*, and *Cdx4* are expressed in overlapping domains with Hox genes in the posterior embryo through E12.5 and that consensus Cdx response elements are found in the promoter regions of several Hox loci. Furthermore, RA was demonstrated to induce *Cdx1 in vivo*, and a RARE was determined thus demonstrating *Cdx1* to be a direct RA target.[59] As some Hox genes contain Cdx-binding motifs, these data also support a role for Cdx members in some Hox genes expression. Subsequent work by this group confirmed an autoregulatory loop for *Cdx1* expression and further demonstrated that in addition to response to retinoid signaling, there is an interaction with the Wnt signaling pathway. Wnt nuclear effectors lymphoid enhancer factor 1 (LEF1) and T-cell factor 1 (TCF1) were demonstrated to interact with the *Cdx1* promotor in association with Cdx to form a Cdx1-LEF1 transcription complex to mediate *Cdx* expression in presomitic mesoderm. Taken together, these data indicate that LEF1 and TCF1 are the principal mediators of *Cdx1* expression in the caudal embryo.

In recent experimentation, the three RARs display distinct expression patterns at day E8.5 with RARα strongly expressed in neuroectoderm and mesenchyme of the head region; RARγ strongly expressed in the tail region; and RARβ transcripts found in the trunk tissues and mesonephric duct. These investigators conclude that the primary target of RA-induced cranio-facial teratogenicity is the branchial arch endoderm and that it is mediated by RARβ/RXR heterodimers in which the activity of RXR is subordinated to that of RARβ.[60]

26.11 SUMMARY AND CONCLUSIONS

Retinoids are small ligands that have been demonstrated to produce biological, pharmacological, and toxicological effects via numerous mechanisms. They clearly and most importantly act as ligands for their nuclear receptors RAR and RXR and by this mechanism are involved in transactivation of a large number of genes, many of which are of documented importance in functioning and malfunctioning of skin, eye, and developing embryo. Through interactions with their receptors and signal transduction pathways they may also be involved in gene transrepression.

Retinoid action in vision is also ligand dependent, producing the signal interpreted by the brain as vision through 11cRAL liganded to opsin in the complex rhodopsin and acting as a GPCR in initiating the signal that is propagated by its unique signal transduction pathway to the optic nerve. The visual cycle also provides an additional putative mechanism of action for retinoids as competitive enzyme inhibitors in their inhibition of the isomerases and dehydrogenases involved in local generation of the ligand 11cRAL.

The role of retinoids in inducing apoptosis is likely of importance in therapeutic interventions for tumor treatment or prevention and perhaps in the developing embryo, and may be a further mechanism of action although fewer specifics for this role *in vivo* are available.

These diverse and somewhat disparate observations do not yet allow definition of a single or unified mechanism of biological action and toxicological effects for retinoids. One very simple fact, however, must always be kept in mind irrespective of the precise mechanism operative in any particular circumstances: retinoids administered in therapeutic doses will always result in sustained, high (up to several hundred-fold higher than any endogenous retinoid) plasma and probably tissue levels of potentially agonistic or antagonsitic ligands. It is probably not possible to adequately regulate these high sustained levels by systemic and local metabolism or normal feed back control mechanisms. In these circumstances, some degree of undesirable effects is with currently available retinoids, nearly a certain corollary of potent therapeutic effect.

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27 Mechanisms in Cutaneous Drug Hypersensitivity Reactions

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27.1 INTRODUCTION

Adverse drug reactions involving the skin are a common problem but their real incidence is not known. Among inpatients 2–5% experience a cutaneous adverse drug reaction (CADR),[1,2] but although it is a frequent cause of consultation or urgent observation at a Dermatology department,[3] no precise data exist concerning its incidence in outpatients. Only 2% are severe reactions,[1] therefore most are not reported to the pharmacovigilance systems. Also, in some cases, the diagnosis of CADR is one of presumption, with no definitive test to prove it, or of exclusion for which the dermatologist has to be alert. Some CADR are mild and resolve spontaneously, others represent an exaggeration of the drug pharmacological effect, some are similar to viral exanthems or idiopatic urticaria, and others mimic skin diseases which are not usually drug-induced, namely pemphigus, bullous pemphigoid, lupus erythematosus, psoriasis, and lichen plannus.[1,3,4]

27.2 PATHOMECHANISMS IN CUTANEOUS ADVERSE DRUG REACTIONS

27.2.1 Immune and Nonimmune Mechanisms Involved in CADR

Most CADR are certainly not immune mediated, representing a pharmacological drug effect often exaggerated due to drug interactions, concomitant diseases that modify drug bioavailability, or predisposing genetic polymorphisms of drug detoxifying enzymes. As an example, skin and oral mucosa erosions can occur during metothrexate treatment in patients with low serum albumin, low renal clearance, or on concomitant use of nonsteroidal antiinflammatory drugs (NSAID). These predictable reactions, called type A, may represent up to 80% of CADR[4] and are not the object of this chapter.

Unpredictable, idiosyncratic CADR, called type B, namely drug "rashes" or "drug eruptions," are those mostly dependent on immune hypersensitivity reactions. CADR upon systemic drug exposure include a wide variety of skin reaction patterns occurring either immediately upon exposure or with a delay of hours, days, sometimes after weeks, or months of drug administration, depending on the hypersensitivity mechanisms involved and whether the individual is already sensitized or not. The pathogenic mechanisms involved are not usually simple and include a complex interplay of different effectors of the immune system, which orchestrate the immuno-inflammatory skin reaction in a still not yet fully understood way.[1,4]

The four classical mechanisms of immune hypersensitivity defined by Gell and Coombs participate in CADR. Immediate type I hypersensitivity from drug-specific IgE is involved in acute urticaria and anaphylaxis, type II antibody-mediated cytotoxicity is reported in drug-induced hemolytic anemia and drug-induced immune complexes can be deposited in small cutaneous vessels inducing leukocytoclastic

vasculitis, representing a type III reaction.[4] Delayed type IV hypersensitivity involving T cells that specifically recognize the drug (or a drug metabolite) has been well documented namely in generalized maculopapular exanthema (MPE), in the drug-induced hypersensitivity syndrome (DIHS) also known as DRESS (drug-induced rash with eosinophilia and systemic symptoms), in acute generalized exanthematic pustulosis (AGEP), in fixed drug eruption (FDE), and in the more widespread and severe Stevens–Johnson syndrome (SJS) or toxic epidermal necrolysis (TEN).[4–6] For each of these different clinical and histological patterns, delayed hypersensitivity is involved through different subsets of T cells and soluble effectors that recruit a wide range of other cells and orchestrate the inflammatory response, inducing lesions that affect only the skin or, eventually, also other organs.[5]

Topical drugs cause allergic contact dermatitis, a typical T-cell mediated reaction, which can become widespread simulating a drug eruption due to percutaneous drug absorption.[6,7] Also, systemic exposure to drugs to which patients were previously sensitized through the skin can induce systemic contact dermatitis,[8] presenting as a "baboon syndrome" also known as SDRIFE (symmetrical drug-related intertriginous and flexural exanthema)[9] or an acrovesicular dermatitis.[6,8]

Photo-active drugs, either upon topical or systemic exposure, can induce a photosensitive eruption on sun-exposed areas, due to a phototoxic or a photoallergic mechanism, in this case dependent on a type IV hypersensitivity reaction to the drug or a photoproduct. One such example is piroxicam, an NSAID which upon UVA exposure gives rise to a photoproduct chemically and antigenically similar to the thiosalicylate moiety of thiomersal, and, therefore, induces photoallergy in individuals previously sensitized to thiomersal.[10,11]

In some cases, the drug modifies the immune response promoting autoimmunity or induces the production of pathogenic antibodies directed against skin structures, as in vancomycin-induced linear IgA dermatitis,[12] in drug-induced pemphigus, and in terbinafine-induced subacute lupus erythematosus. Sometimes autoantibodies are found during the eruption but their pathogenic significance is not known, as in a case of DRESS with antibodies against the 190 kDa antigen targeted usually in pemphigus foliaceus or paraneoplastic pemphigus.[13]

27.2.2 Drug Recognition by the Immune System and Skin Reaction Patterns

Apart from the wide list of drugs capable of inducing immune-mediated CADR, each drug can induce several skin reaction patterns and depending on the reaction pattern (and sometimes within the same reaction pattern), the antigenic moiety recognized by the immune system is different: the drug itself, an intermediate metabolite, both the drug and a metabolite, or proteins/peptides modified by reactive drugs or metabolites. Drugs can be specifically recognized by IgE in immediate hypersensitivity, by antibodies fixed on red blood cells inducing hemolytic anemia, by soluble antibodies inducing immune complex vasculitis, or by the TCR of T cells, both in the context of human leukocyte antigen (HLA) class-I or class-II molecules and either covalently or non-covalently bound.[14,15] The problem is more complex as, for instance, in the case of MPE from cotrimoxazol. Some T-cell clones recognize sulfamethoxazole and other antiinfectious sulfonamides with a similar conformational structure, giving rise to cross-reactions,[16] whereas other T-cell clones recognize intermediate metabolites (hidroxilamine sulfamethoxazole or nitroso sulfamethoxazole,[15]) either presented directly in the HLA groove[14,15] or after antigen processing[14,16] with a more restricted cross-reactive pattern. In the case of immediate hypersensitivity to penicillin, IgE can recognize either the benzylpenicilloyl moiety or the side chain; therefore, recognizing by cross-reaction other penicillins or, eventually, also cephalosporins.[17,18] For piroxicam, the moiety recognized by the immune system depends on the reaction pattern. The thiosalicylate moiety, which is formed after UVA radiation, is responsible for the photoallergic reaction. As this photoproduct is exclusive for piroxicam, other oxicams like tenoxicam can be safely used in photoallergy. In FDE the immune system recognizes another oxicam moiety which is common to tenoxicam and, therefore, all patients with FDE to piroxicam cross react with tenoxicam.[10,11,19]

27.2.3 Concomitant and Predisposing Factors in Drug Eruptions

Even though we do not understand all the steps of sensitization to drugs and how some individuals become sensitized and develop CADR while others do not, there are few known predisposing factors.

One important aspect deals with the drug detoxification process where polymorphisms within drug metabolizing enzyme genes, namely in the cytochrome P_{450}, can give rise to different intermediate reactive (or nonreactive) drug metabolites or to distinct amounts of the culprit metabolite.[20,21] Some HLA haplotypes, which may be related to the capacity of the drug to combine or insert into the HLA groove of antigen presenting or target cells, have been associated with increased or reduced capacity to develop a drug eruption to a certain drug,[5] as shown for HLA-B*1502 predominance in patients from Twaian who develop SJS to carbamazepine.[22] Also, polymorphisms in immuno-inflammatory response pathways may increase the risk of some particular drug reactions: predisposition to produce higher levels of soluble FAS ligand and polymorphisms in the TNF-promoter region may correlate with an increased severity of drug reactions,[5,22] disturbances in complement and cinin metabolism, namely in carboxypeptidase that degrades bradykinin, may favor angioedema induced by angiotensin conversing enzyme inhibitors (ACEI), and polymorphisms in the gene for LTC4 synthase may justify familial aggregation of aspirin-induced urticaria.[23]

Also, the immune status of the patient during drug exposure may be important for the outcome of the CADR. Concomitant aggressions (exposure to other reactive chemicals or other drugs), infectious diseases (bacterial or viral infections),

chronic immuno-inflammatory diseases (Still's disease, systemic lupus erythematosus), or nonspecific immune activation by reactive drug metabolites may act as "danger" signals that alert the innate immune system and activate monocyte/macrophages or dendritic cells that become increasingly capable of presenting the drug to T cells.[20,24] Therefore, these concurrent factors may be of extreme importance, especially during active drug sensitization, but also during the development of the CADR in a sensitized individual. As an example, patients with systemic lupus erythematosus or HIV+ patients are more susceptible to CADR, namely from sulfonamides.[15,24] During Epstein-Barr virus (EBV) or Cytomegalovirus (CMV) infection, antibiotics induce MPE in a high proportion of patients,[25,26] but even though aminopenicillins induce an MPE in almost every patient, only a few of these really become sensitized to the drug and develop a skin rash on re-exposure without the concomitant infection.[25–27] Also, during the last decades attention has been drawn to the association of the DIHS/DRESS with human herpes virus type 6 (HHV-6) primo-infection or reactivation.[26,28,29] Concomitant use of aminopenicillins and allopurinol also seem to represent a risk factor for developing CADR.[25,30]

Nevertheless, and apart from these difficulties and variables that complicate the study of pathomechanisms involved in CADR, immediate and delayed skin testing, drug rechallenge, and *in vitro* studies using drug-specific antibodies or drug-specific T cell clones isolated from the blood and skin of patients with CADR or from positive skin tests, have brought new light into the immune mechanisms involved in CADR, which we will review for the main reaction patterns.

27.3 IMMEDIATE ADVERSE DRUG REACTIONS

These reactions occur within minutes to a few hours after drug exposure and present clinically as pruritus, urticaria, or angioedema regressing with no residual lesions within minutes to hours. In severe cases, urticaria and angioedema are associated with systemic symptoms like nausea, abdominal cramps, sneezing, bronchospasm, and dispnea that can progress to hypotension and shock in its most severe expression—anaphylaxis. The most severe acute immediate reactions are induced by beta-lactam antibiotics (pencillin G and aminopenicillins), iodinated radiocontrast media, and muscle relaxants used in anesthesia, whereas the more frequent but less severe immediate reactions are due to aspirin and NSAID, codein, vancomycin, ACEI, heparins, and insulin, but any drug can induce an immediate adverse reaction.[4,17,31] (Figure 27.1 shows an immediate reaction with urticaria and angioedema from an NSAID.)

Immediate reactions are dependent on drug-specific IgE fixed on tissue mast cells and circulating basophils, but clinically similar reactions, although usually less severe, occur without the identification of a drug-specific immune reaction, and are, therefore, called pseudoallergic or anaphylactoid.[1,4,31] In all cases, the tissue mast cells, blood basophils, and, eventually, platelets[32] liberate the content of their granules (histamine, tryptase, heparin, cytokines, and chemokines) and produce secondary vasoactive mediators (prostaglandins, leukotrienes, PAF/platelet activation factor, and cinins), which together are responsible for the vasodilatation, increased vascular permeability, and pruritus observed in urticaria.[4,33]



FIGURE 27.1 Angioedema and urticarial lesions after ingestion of an NSAID (diclofenac).

In immediate hypersensitivity, cell degranulation occurs upon specific mast cell or basophil IgE bridging by the drug.[4] Nevertheless, degranulation can occur by non-IgE-dependent mechanisms like the activation of cell receptors for complement anaphylotoxins (C3a and C5a), direct drug effect on the cellular membrane or in intracellular pathways that regulate degranulation, or imbalance between prostagladins and leukotrienes due to cyclooxygenase inhibition by NSAID. Still, a similar reaction can occur from the increase of bradikinin and other vasoactive mediators due to drugs that inhibit their degradation, like ACEI.[1,4,34–36]

In immediate hypersensitivity reactions, a drug specific IgE is found in *in vitro* tests, there is *in vitro* drug specific basophil activation (measured either by the expression of CD63 by flow cytometry or by mediator release),[33,37] and immediate skin testing (prick or intradermal) and drug rechallenge (which is not advised in severe cases) are positive in a high proportion of patients (>80%).[31] Nevertheless, with several drugs that induce IgE-mediated reactions, like muscle relaxants, iodinated radiocontrast media, and heparins, there is also a direct capacity for nonspecific basophil or mast cell activation, which can be responsible for nonspecific positive skin and basophil activation tests (CD63 expression

or mediator release).[34,35,38] Also, occasionally, drug-specific IgE has been documented in aspirin-induced urticaria and asthma, classically considered pseudoallergic,[39] and even for penicillin a nonspecific capacity for mast cell activation (albeit low) has been documented *in vitro*. Therefore, this makes the distinction between what are called allergic and pseudoallergic reactions difficult, both on clinical and laboratory grounds.

Drug-specific IgG or IgM antibodies can also be responsible for immediate symptoms,[36] because these antibodies give rise to circulating immune complexes and complement activation and induce urticaria with systemic symptoms within the context of serum disease (fever, arthralgia or arthritis, abdominal pain and urticaria, urticaria vascultis, or leukocytoclastic vasculitis), which occurs either immediately or within a few days of drug administration.[4,40]

27.4 DELAYED CUTANEOUS ADVERSE DRUG REACTIONS

There are several clinical and experimental arguments that confirm the involvement of delayed type hypersensitivity with the participation of drug-specific T cells in the following CADR: MPE, DIHS/ DRESS, AGEP, SJS, TEN, and FDE.[4,24,41] (1) These eruptions begin within 7–21 days in the 1st episode and 1–2 days after drug reintroduction; (2) drug-specific positive oral rechallenge with lower doses is usually observed;[42] (3) on histopathology there is mainly a dermo-epidemal infiltration of activated T cells; (4) in a high percentage of cases, the culprit drug induces specific positive patch, prick, or intradermal skin testing with delayed readings;[6,43–46] (5) *in vitro* tests show drug-specific T lymphocyte proliferation/activation;[47,48] and (6) drug-specific T-cells lines and T-cell clones have been isolated from the blood and skin during the acute episode or, later, from positive patch tests.[41,49]

Nevertheless, as there are distinct subsets of T cells with distinct cytokines/chemokines and aggressive machinery, they orchestrate the inflammatory skin reaction giving rise to different patterns of drug reactions. Therefore, a subdivision of delayed hypersensitivity T-cell reactions has been made in agreement into type IVa, IVb, IVc, and, more recently, type IVd.[5] They represent, respectively, the reactions mediated predominantly by T-helper 1 [interferon (IFN)-γ], T-helper 2 [interleukin (IL)-4 and IL-5], cytotoxic reactions (CTL, CD8+ rich in perforin, granzyme B, and FasL), and CXCL8 (IL-8) secreting T cells that promote neutrophilic inflammation.[24,50]

The participation of these subsets is very particular in the different patterns of delayed drug eruptions, as detailed in Table 27.1.

27.4.1 Maculopapular Exanthems

MPE, the most frequent pattern of CADR, appear as generalized symmetric eruptions of isolated and confluent erythematous macules or papules, often starting in the trunk and then spreading to the extremities. Mucosa are not involved, there are no evident systemic symptoms apart from a low-grade fever which can also contribute to mimic a viral or bacterial exanthem. The reaction develops within

TABLE 27.1
General Aspects of the Hypersensitivity (HS) Mechanisms Involved in the Main CADR

Type of Reaction	Immediate	Delayed				
Reaction pattern	Urticaria/ anaphylaxis	MPE	DRESS	AGEP	SJS/TEN	FDE
Main drugs	Penicillins, Contrast media, NSAID	Antibiotics Anticonvulsivants Allopurinol	Anticonvulsivants Allopurinol Minocycline	Antibiotics Aminopenicillins	Allopurinol Anticonvulsivants Sulfonamides	NSAID
Drug recognition	IgE	TCR/HLA I-II	TCR/HLA-II	TCR /HLA I	TCR	TCR
Effector cells	Mast cells Basophils	CD4/CD8	CD4/CD8 eosinophils	CD4+ neutrophils	CD8+CD56+	CD8+CD69
Soluble mediators	Histamine Tryptase PGs, LTs, PAF	Perforin IFN-γ IL-5	IL-5 IFN-γ	CXCL8 GM-CSF IFN-γ	Fas/FasL TNF-α Perforin	Fas/FasL IFN-γ
Target cells	Endothelial cells	Keratinocytes Other skin cells	Keratinocytes Other skin cells	Epidermis	Keratinocytes	Keratinocytes
In vivo tests	Prick/idr	Patch testing Oral challenge	Patch testing	Patch testing	…	Lesional testing Oral challenge
In vitro tests	Specific IgE Basophil activation	LTT	LTT	LTT	LTT	N/A
Other aspects	Similar to pseudoallergic reactions	Mimic viral and bacterial exanthems	Concomitant HHV-6	Neutrophilic Inflammation	High mortality rate	"preactivated" T cells in residual lesions
Type of HS	Type I	Types IVa, IVb, and IVc	Types IVa, IVb, and IVc	Type IVd	Type IVc	Type IVc



FIGURE 27.2 Maculopapular exanthem from carbamazepine.

7–14 days after drug intake (or within 1 or 2 days in sensitized patients) mainly due to antibiotics (aminopenicillins, cefalosporins, and sulfonamides), allopurinol, and anticonvulsivants. The reaction may be mild and regress within a few days, but most often it progresses for a few days even after drug suspension and then fades progressively within 10–15 days, often with desquamation.[5,51] (Figure 27.2 shows an MPE from carbamazepine.)

On histopathology, early lesions show an interface dermatitis with hydropic degeneration of basal keratinocytes, mild spongiosis, scattered dyskeratotic and necrotic keratinocytes, and lymphocytes mainly at the dermal epidermal junction and papillary dermis with eosinophils along dermal vessels.[24,51,52] Lymphocytes are skin homing highly activated T cells (CLA+, CD3+, DR+, CD25+) expressing adhesion molecules such as CD11a-CD18 (LFA-1) and CD62L (L-selectin). They are attracted from the blood through the expression of the corresponding adhesion molecules in endothelial cells and keratinocytes (ICAM-1, HLA-II) and by the production of. keratinocyte chemokines, like CCRL27 (also known as CTACK-cutaneous T-cell attracting chemokine) that selectively recruits skin homing memory T cells expressing the CCR10 receptor.[15,52,53] Most lymphocytes infiltrating the skin are CD4+ T cells expressing high levels of perforin and granzyme B but CD8+ T cells are also found, mainly in the epidermis.[5,51,52] T cells secrete a heterogenous profile of cytokines and chemokines: type 1 cytokines (IFN-γ) activate dendritic cells and keratinocytes increasing their expression of HLA-II that binds the drug and presents it to T cells; IL-5, a type 2 cytokine, along with the eotaxin/CCL-11 is responsible for the recruitment and activation of eosinophils, a local and systemic hallmark of cutaneous maculopapular drug eruptions.[51] During the acute phase, CLA+CD4+ T cells expressing perforin are also increased in the blood and after isolation exhibit *in vitro* cytotoxic activity against keratinocytes, therefore reinforcing their capacity to cause keratinocyte damage in the skin.[51] Similar cells have been isolated from positive epicutaneous patch tests with the culprit drug and it has been shown that T-cell clones isolated from the blood, skin, and positive patch tests in patients with MPE are specifically stimulated by the culprit drug and exhibit similar profiles of activity, namely perforin expression and production of cytokines and chemokines (INF-γ, IL-5).[41,49]

Therefore, after a process of T-cell sensitization, a further exposure to the drug that reaches the skin and combines with skin proteins or HLA molecules of keratinocytes and dendritic cells activates resident skin and circulating CD4+ and CD8+ T cells, which are attracted to the skin and selectively damage the cells where the drug is fixed, mainly by perforin and granzyme B. Cytokines and chemokines produced by T cells and resident skin cells recruit other inflammatory cells that orchestrate the dermal and epidermal inflammatory reaction in MPE. Therefore, various subtypes of delayed hypersensitivity, mainly types IVa, Ivb, and IVc, seem to be involved in this pattern of CADR.[5]

27.4.2 Drug-Induced Hypersensitivity Syndrome/Drug Reaction with Eosinophilia and Systemic Symptoms

DRESS is a severe life-threatening CADR that develops 2–8 weeks after drug intake, usually an anticonvulsivant, allopurinol, a sulfonamide, dapsone, or minocycline. It involves the skin, presenting with a nonspecific maculopapular rash or a more generalized exfoliative dermatitis, often with severe facial edema (Figure 27.3 shows a case of DRESS induced by allopurinol with an exfoliative dermatitis and facial edema). Systemic symptoms are always present and consist of fever, malaise, arthralgia, enlarged lymph nodes, hepatic, renal, or pulmonary failure. Leukocytosis with circulating atypical (activated) lymphocytes occurs with eosinophilia that may appear a few days later. It begins after a longer interval than for other drug rashes and also regresses slowly often with exacerbations, either related with steroid withdrawal, viral reactivation, or administration of a cross-reactive drug.[1,28,29] Also, delayed reactivation apparently with no drug exposure or with exposure to a nonrelated drug has been reported.[54]

In DRESS, circulating activated T cells expressing CLA+ and CCR10 increase in the blood in proportion with the skin severity, and these CD4+ and CD8+ T cells infiltrate the dermis and epidermis.[15] In carbamazepine and lamotrigine sensitive patients, T-cell clones generated from these infiltrating skin and circulating cells react specifically to these drugs on HLA-II matched antigen-presenting cells



FIGURE 27.3 Exfoliative dermatitis with facial edema in a case of DRESS induced by allopurinol.

apparently independent of drug metabolism and antigen processing.[15] Most of these T cells share the TCR Vβ 5.1 chain, suggesting that the drug might also act as a superantigen.[55] These T-cell clones are rich in perforin and secrete a type 1 cytokine pattern with IFN-γ and chemokines that control the duration and severity of the inflammatory response.[15] They also show a very significant IL-5 secretion which is responsible for the characteristic eosinophilia observed in this syndrome.[56]

Nevertheless, and even though these drug-specific T-cells clones have been isolated in DRESS[15] and, in our experience, patch tests with the drug, namely with carbamazepine, are often positive,[57] the pathomechanisms involved seem to be complex and not exclusively dependent on the drug. Most authors refer the need for a concomitant HHV-6 reactivation, which would be responsible for the systemic symptoms as well as for exanthem reactivation without drug.[29,28,54] Recent studies presented by Yoko Kano and Testsuo Shiohara suggest that HHV-6 reactivation, evaluated by detection of viral deoxyribonucleic acid (DNA) by polymerase chain reaction (PCR) and by the increase in anti-HHV-6 IgG titer in blood, occurs after a certain degree of immunossupression, particularly hypogammaglobulinemia, induced by the drug.[29] They also suggest that, just after drug suspension, the recovery of CD4+ and CD8+ cells will be responsible for an immune reconstitution inflammatory syndrome (IRIS) with damage of the tissues where the virus/drug is localized, as observed in acquired immune deficiency syndrome (AIDS) after highly active antiretroviral therapy (HAART) treatment.[58] This might explain why, in their experience, lymphocyte transformation tests (LTT) are positive only after a certain time of evolution of the DRESS when there is a full immune reconstitution.[58]

Although drug-specific T cells with a high production of IL-5 and eotaxin, responsible for the systemic and skin eosinophilia,[55,56] have been observed in DRESS, suggesting the involvement of a type IVb and IVc hypersensitivity reaction, further studies are needed to fully understand the mechanisms underlying this severe ADR.

27.4.3 Acute Exanthematic Generalized Pustulosis

AGEP is a very peculiar reaction pattern induced by drugs in more than 90% of cases, mainly by aminopenicillins and other antibiotics. It is characterized by the acute onset of symmetrical widespread edematous erythema covered by small nonfollicular sterile pustules, predominating in the face and body folds, high fever (>38°C), leukocytosis, neutrophilia, and, occasionally, eosinophilia. (Figures 27.4a and 27.4b show a patient with AGEP from amoxicillin with the predominance of small pustules on body folds.) The reaction develops around 1 week after drug intake and regresses in 5–10 days after drug withdrawal. Lymphocyte transformation tests and, typically, patch tests are positive[9,59] and, after 72 hours, show a pustulous pattern similar to the acute reaction.[60,61]

The histology and immunohistochemistry of early biopsies from AGEP show a dermo-epidermal infiltration of T cells, mainly CD4+DR+CD25+, with discrete vacuolar keratinocyte degeneration and a perivascular infiltrate, sometimes with vasculitis.[62,63] Lesions progress to spongiotic vesicles that soon transform into subcorneal pustules due to neutrophil accumulation.[63] This same pattern occurs at positive patch tests, which make them a very useful tool to study the pathomechanisms involved in AGEP. From the blood and skin biopsies of patch tests, several drug-specific T-cell lines and T-cell clones have been isolated and characterized. They are mainly CD4+ memory effector T cells, which exhibit cytotoxicity against drug laden target cells, both through perforin/granzyme B and Fas ligand.[64] They secrete mainly a type 1 cytokine pattern (IFN-γ and Granulocyte-macrophage colony-stimulating factor (GM-CSF)), in some cases with IL-5, responsible for eosinophilia observed in about one-third of AGEP patients.[61] Nevertheless, the main particular characteristic of these T cells is the high production of CXCL8 (IL-8) and GM-CSF, that recruit and prolong survival of neutrophils in the skin. Actually, *in vitro* tests have shown that apart from CXCL8 that recruits neutrophils bearing the CXCR1, other mediators of these T cells, like GM-CSF and INF-γ, acting mainly through the CXCR2, prevent neutrophil apoptosis and prolong their skin survival.[60]

But, preceding neutrophil skin infiltration, drug-specific CD4+ T cells (with less than 30% CD8+), expressing CCR6 as the skin homing receptor, are present in the skin and exert some cytotoxicity in the epidermis[5] before they secrete

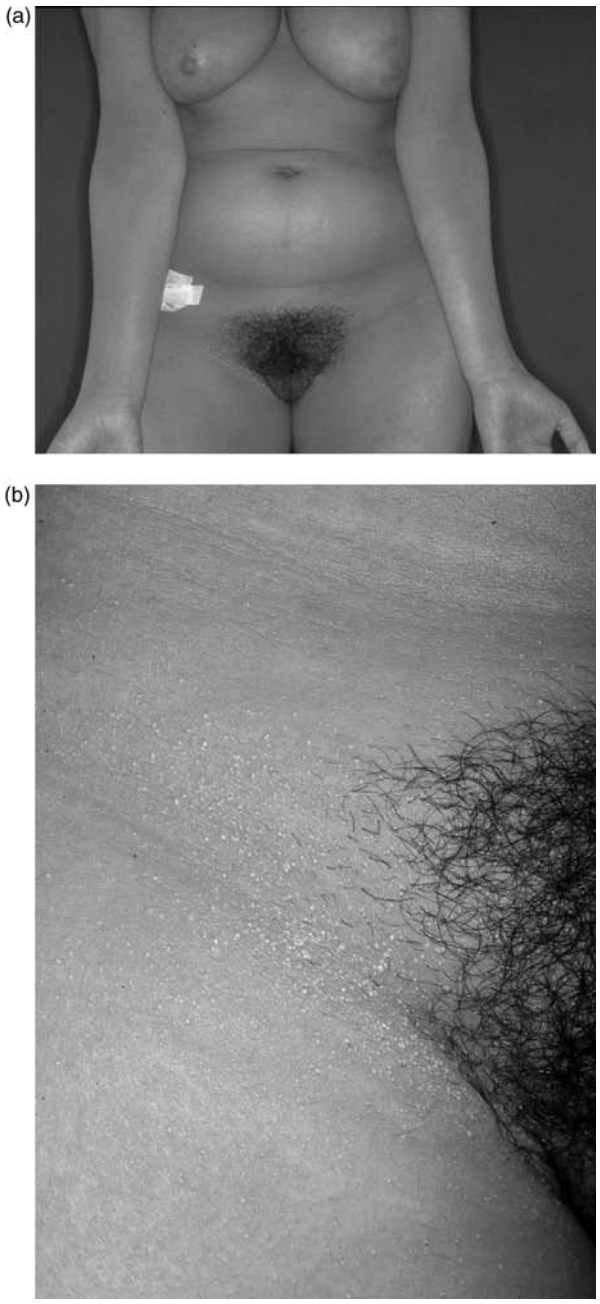


FIGURE 27.4 (a) Acute generalized exanthematic pusutulosis induced by amoxycillin. (b) Detail of Figure 27.4a. Small pustules mainly on body folds in AGEP.

CXCL8 that recruits neutrophils. As both T cells and keratinocytes secret CXCL8 and T cells also express the CXCR1, there is further T-cell activation by CXCL8 produced by keratinocytes.[61] Opposing MPE, there is a much lower expression of HLA-II by keratinocytes and no exotaxin was observed in the epidermis, but only along endothelial cells.[61]

This very peculiar pattern of drug-specific T-cell reaction, now considered a type IVd hypersensitivity reaction,[5] develops with drugs that usually induce other type IV reactions. No reason has, thus far, been found to justify why in some patients and in what circumstances a drug can elicit this particularly CXCL8 rich T-cell activity.

27.4.4 Stevens–Johnson Syndrome/ Toxic Epidermal Necrolysis

SJS and its more extensive variant, TEN, represent a life-threatening pattern of CADR characterized by widespread symmetrically distributed macular lesions, showing typical or mainly atypical targets, with central bulla, that coalesce to form large sheets of necrotic epidermis covering more than 30% of the body surface area in TEN (Figure 27.5 represents a case of TEN from allopurinol, with skin detachment involving about 60% of the body surface area). The eruption is often preceded by fever, malaise, mucosal pain/erosions and, as the skin rash progresses from the head to the extremities, fever and systemic symptoms occur in a variable intensity and combination. Conjunctivae, oral, and genital epithelial shedding is usually intense and painful, and can be associated with epithelial necrosis of the oropharynx, gastrointestinal tract, trachea, and bronchia. SJS/TEN are due to drugs in more than 90% of cases, usually an antibiotic (sulfonamide), allopurinol, an anticonvulsivant (lamotrigine, carbamazepine), or an NSAID (oxicam).[1,4] In the skin there is a variable accumulation of inflammatory cells, ranging from an almost absent to a dense dermal T-cell infiltration, which seems to correlate positively with the area of skin detachment and, consequently, with the mortality rate.[65,66] Factor XIIIa+ dermal dendritic cells are increased contrasting with a reduction of CD1a+ Langerhans cells. CD4+ and CD8+ T cells are scattered in the dermis and many cytotoxic activated CD8+CD56+ T cells are found in the blister fluid.[67,68] But the most striking histologic marker of TEN is the keratinocyte cell death extending to all epidermal layers.[65] There is evidence that this is due to apoptosis, dependent on several mechanisms. The Fas/Fas ligand (CD95/CD95L) pathway, in its membrane bound or soluble form, seems to be mainly involved, but there are other pathways leading to keratinocyte apoptosis, namely TNF-α, granzyme B, and perforin- and calcium-dependent calprotectin.[69–71] These soluble mediators are found in high amounts in the serum but very particularly in the blister fluid, where they are detected with other cytokines (IL-18, IFN-γ, and IL-10) liberated by damaged keratinocytes which amplify the inflammatory loop and epidermal apoptosis.



FIGURE 27.5 Extensive skin detachment in a patient with toxic epidermal necrolysis from allopurinol.

The main origin of these death mediators are drug-specific T cells, mainly CD8+, present in the blister fluid of patients with TEN.[67,68] These CD8+CD56+ T cells have an important cytotoxic potential against HLA-I restricted keratinocytes combined with the culprit drug, mainly due to granzyme B perforin,[16,68] and soluble Fas produced in high amounts after drug stimulation.[70] Therefore, after a first aggression by CD8+ cytotoxic T cells that need cell contact or proximity, other soluble mediators secreted by drug-specific T cells (IFN-γ, sFas) can be important for disease spreading. IFN-γ activates keratinocytes that increase HLA-I expression, rendering them more susceptible to CD8+ specific T-cell killing.[16] It upregulates keratinocyte secretion of CCL27/CTACK, that further attracts CCR10+ cutaneous memory T cells,[53] and increases their expression of receptors for TNF and Fas and their production of Fas ligand, making keratinocytes more susceptible to apoptosis and capable of inducing apoptosis of neighboring cells.[16]

The factors that drive the CADR into an SJS or TEN are not known. TEN inducing drugs are not different from those that induce other CADR, and sometimes at the beginning the skin reaction simulates an MPE. Nevertheless, increased serum levels of soluble Fas may indicate the progression to a more severe life-threatening reaction,[70] and some authors suggest that, in individuals who develop SJS or TEN, their lymphocytes have an increased capacity of secreting sFas, even in basal conditions.[22] Therefore, this and other genetic susceptibility markers can be of importance in determining this pattern of CADR.

27.4.5 Fixed Drug Eruption

FDE is due to drug hypersensitivity in more than 95% of the cases. The clinical presentation is very typical, with round erythematous lesions, that may progress to plaques or bulla and regress spontaneously within 10–15 days with a grey-brown hyperpigmentation (Figure 27.6 shows two typical round lesions of FDE induced by piroxicam). Lesions may vary from a few to a widespread involvement making a differential diagnosis with TEN difficult.[19,72]

At the acute phase there is a mononuclear inflammatory infiltrate, mainly at the dermal epidermal junction, with hydropic degeneration of basal keratinocytes and scattered or more extensive apoptosis of keratinocytes, eventually involving the whole epidermal thickness, as in TEN. Upon regression, melanophages are easily visible in the dermis for years and, if special immunohistochemical stains are performed, CD8+ T cells can also be detected in the epidermis in abnormal numbers over prolonged periods after clinical resolution.[74] This is probably due to the expression of the skin homing receptor (CLA+) and the integrin α3β7 (CD103), which binds E-cadherin in keratinocytes.[73,75] These CD3+, CD45RA+, CD11b+, CD8+ effector memory T cells share some surface and activation markers with NK cells, namely the CD69,[73,75] but in the residual phase they do not harm the neighboring cells, which are protected from apoptosis.[74] Within a few hours upon exposure to the culprit drug these resting or "pre-activated" T cells initiate a process of epidermal aggression. They upregulate mRNA for IFN-γ and secrete this cytokine in high amounts;[73,74] they express Fas ligand which binds Fas on keratinocytes, thus inducing apoptosis;[73–75] TNF-α, perforin, and granzyme secreted by these cells and other CD8+ effector T cells recruited from the circulation also participate in the epidermal aggression.[73,74] Along with CD8+, which migrate mainly to the epidermis, CD4+, localize preferentially in the dermis. Among these CD4+CD25+hi regulatory T cells seem to downregulate the reaction either by direct cell contact or by secretion of IL-10 or TGF-β.[76] These cells also seem to be involved in the process of desensitization in FDE.[77]

The presence of the "pre-activated" T cells in the residual lesional epidermis can explain why patch testing is negative in normal skin, whereas a few hours after application of the culprit drug in a residual lesion reactivation occurs with the clinical and histhopathology typical of an FDE.[19,72] Although some authors suggest that these lesions can be reactivated by nonspecific stress/danger signals,[74,78] in our experience lesional reactivation by patch testing is drug specific and allows the confirmation of the culprit drug and study of cross reactions.[19,79]



FIGURE 27.6 Typical round erythemato-violaceous lesions in fixed drug eruption from piroxicam.

27.5 CONCLUDING REMARKS

The knowledge of the pathomechanisms involved in drug hypersensitivity is of extreme importance for the clinician to understand the clinical and evolutive pattern of CADR, to choose the most adequate therapeutic attitude when facing a CADR, to understand and determine which drug is imputed with the highest probability in patients on multiple therapies, to further choose the most adequate complementary tools to confirm the culprit drug (immediate skin tests and IgE/basophil activation tests in immediate reactions, patch testing, or IDR with late readings and LTT in delayed reactions), and to take the most adequate preventive measures to avoid a further CADR.

Nevertheless, several aspects of these mechanisms are not yet fully understood, namely what triggers sensitization to the drug, which drug epitopes (or other related epitopes) are recognized by the immune system so that cross reactions are better previewed and patients are better informed

on drugs to avoid in the future, how lesions fully develop and how we can interfere in their progression, at least in the most severe reactions like SJS and TEN for which no therapy can significantly reduce mortality.

Also, the study of hypersensitivity mechanisms induced by drugs, where oral rechallenge or patch testing has been a complementary tool to understand more pieces of this complex puzzle, contributed to the understanding of pathomechanisms involved in nondrug-related skin diseases. The discovery of CXCL8+ producing T-cell clones in AGEP has stimulated the study of their contribution in other neutrophil rich inflammatory skin diseases, like psoriasis, Sweet's syndrome, and Beçhet's disease, and have given immunologists the suggestion to consider a new type IV hypersensitivity reaction (IVd).[5,60]

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28 Drug-Induced Ocular Phototoxicity

Joan E. Roberts

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Although the human eye is constantly subjected to both artificial and sunlight, damage rarely occurs from this light exposure (Roberts 2005) unless the eye is aged (Roberts 2001; Andley 2001; Balasubramanian 2005) or the light is particularly intense (Sliney 2005). However, many drugs, dietary supplements, cosmetics, and diagnostic dyes have the potential to induce damage to the lens and retina in the presence of ambient light (Fraunfelder and Fraunfelder 2004; Roberts 2004). This danger is enhanced with increased exposure to intense light because of high altitudes (Hu et al. 1989), outdoor employment (Sliney 2001), sun bed use, or phototherapy for seasonal depression (Roberts et al. 1992).

28.1 STRUCTURE OF THE EYE

The structure of the human eye is shown in Figure 28.1. The outermost layer contains the sclera, whose function is to protect the eyeball, and the cornea, which focuses incoming light onto the lens. Beneath this layer is the choroid containing the iris and ciliary body; this layer is known as the uvea. This region contains melanocytes that contain the pigment melanin, whose function is to prevent light scattering. (Hu 2005) The opening in the iris, the pupil, expands and contracts to control the amount of incoming light. Behind the iris is the lens, whose function is to focus light onto the retina. The iris and the lens are bathed in the aqueous humor, a fluid that maintains intraocular pressure; this fluid also contains various antioxidants. Transport to the lens is through the aqueous.

Behind the lens is the vitreous humor, a fluid that supports the lens and the retina and that also contains antioxidants. The retina itself contains the photoreceptor cells (rods and cones) that receive light and the neural portion (ganglion, amacrine, horizontal, and bipolar cells) that transduces light signals through the retina to the optic nerve. Behind the photoreceptor cells are the retinal pigment epithelial cells, Bruchs' membrane, and the posterior choroid. The photoreceptor cells are avascular; their nutrient support

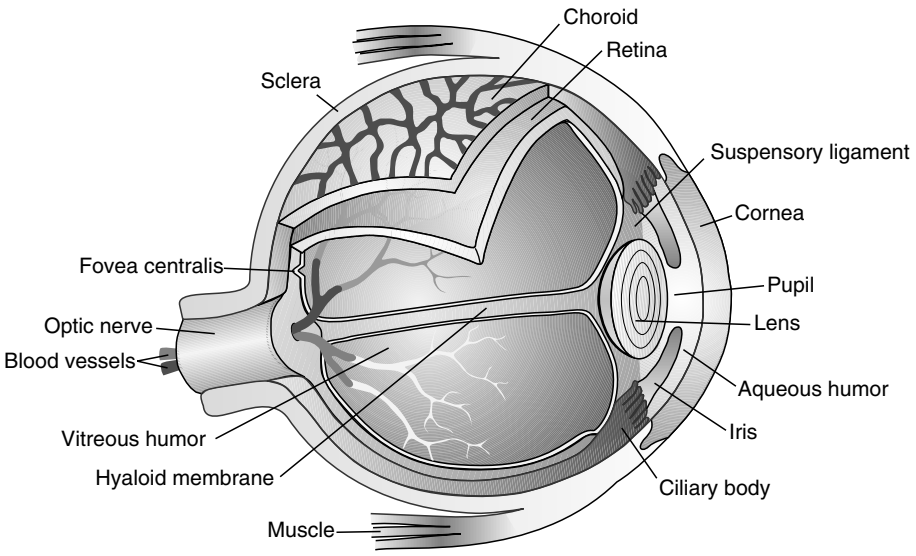


FIGURE 28.1 The structure of the human eye.

(ions, fluid, and metabolites) is provided by the retinal pigment epithelial cells. Transport to the retinal pigment epithelial cells is carried out by the choriocapillaries across the Bruch's membrane.

28.2 TRANSMISSION OF LIGHT THROUGH THE HUMAN EYE

Ambient radiation from the sun or from artificial light sources contains varying amounts of UV-C (220–290 nm), UV-B (290–320 nm), UV-A (320–400 nm), and visible light (400–700 nm). The shorter the wavelength, the greater the energy, and therefore the greater the potential for biological damage. However, although the longer wavelengths are less energetic, they penetrate the eye more deeply (Roberts 2001).

For a photochemical reaction to occur in the eye, the light must be absorbed in a particular ocular tissue. The primate/human eye has unique filtering characteristics that determine in which area of the eye each wavelength of light will be absorbed (Bachem 1956). All light of wavelengths shorter than 295 nm is cut off by the human cornea. This means that the shortest, most energetic wavelengths of light (all UV-C and some UV-B) are filtered out before they reach the human lens (Figure 28.2). Most UV light is absorbed by the lens, but the exact wavelength range depends upon age. The adult human lens absorbs the remaining UV-B and all the UV-A (295–400 nm); therefore only visible light reaches the retina. However, the very young human lens transmits a small window of UV-B light (320 nm) to the retina, while the elderly lens filters out much of the short blue visible light (400–500 nm) (Barker et al. 1991). Transmission also differs with species; the lenses of mammals other than primates transmit ultraviolet light longer than 295 nm to the retina (Barker et al. 1991).

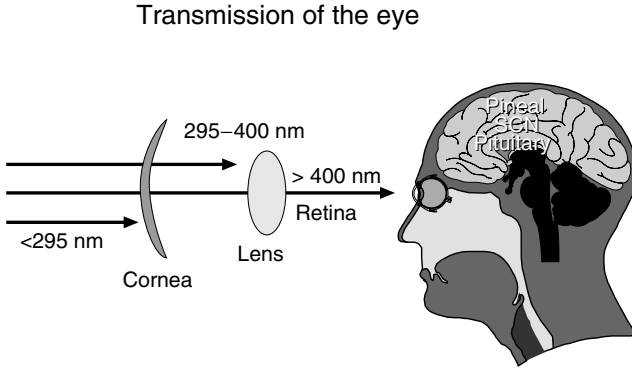


FIGURE 28.2 The eye as an optical system. The cornea filters out light below 295 nm; the adult human lens absorbs light between 295 and 400 nm; and the retina receives light above 400 nm.

28.3 OCULAR PHOTOTOXICITY INDUCED BY XENOBIOTICS

Ultraviolet light (295–400 nm) and very short visible light (400–450 nm) are the source of most of the damage induced in the eye by direct irradiation. However, in the presence of a light activated (photosensitizing) drug, herbal medication (Schey et al. 2000; Taroni et al. 2005; He et al. 2004) or diagnostic dye (Roberts 1981, 1984; Wu et al. 2004) patients are in danger of enhanced ocular injury from both ultraviolet and all wavelengths of visible light (295–700 nm) (Roberts 2004). The extent to which a particular dye or drug is capable of producing phototoxic side effects in the eye depends on several parameters including: (l) the chemical structure; (2) the absorption spectrum of the drug; (3) binding of the drug to ocular tissue; and (4) the ability of the drug to cross blood-ocular barriers.

28.4 SHORT SCREEN FOR PREDICTING POTENTIAL PHOTOTOXICITY

Any compound that has a tricyclic, heterocyclic, or porphyrin ring structure is a potential ocular chromophore. If a drug absorbs ultraviolet light, it may damage the lens, whereas if it absorbs visible light it may also affect the retina. When exogenous sensitizers bind to ocular tissues (i.e., lens proteins; Roberts et al. 1991, 1990), melanin (Borel et al. 2005; Kristensen et al. 1995), and DNA (Martinez and Chignell 1998), their lifetime in the eye is extended and the hazard is enhanced. Substances that are amphiphilic or lipophilic are able to cross most blood-ocular barriers (Roberts et al. 1991).

In summary, there are certain chemical characteristics that allow for the prediction of potential ocular phototoxicity of a substance. These are presented in Table 28.1.

The chemical structure and absorption spectrum of a drug gives the first clear indication of potential phototoxicity. For a chemical compound (prescription drug, herbal medication, diagnostic dye) to induce a phototoxic response in any biological tissue, it must first absorb light. This absorption is limited by the filtering characteristics of the biological tissues involved. The human cornea absorbs all optical radiation below approximately 293 nm (Bachem 1956). In general the lens absorbs most of the radiation between 300 and 400 nm, transmitting light of longer wavelengths to the retina. However, the exact filtering characteristics of the lens vary with age (Figure 28.2) and certain disease states (Barker et al. 1991). Therefore, a drug with an absorbance in the near UV or visible range is a potential photosensitizer of the lens or retina. The most potent photosensitizers usually have structures, which are heterocyclic, tricyclic, or porphyrin-related ring systems (Figure 28.3). They should be amphiphilic or lipophilic to cross blood–brain, blood–retina, and aqueous–lenticular barriers.

Additional information can be obtained by measuring the absorption spectrum of the drug in the presence and absence of lens proteins, DNA, or melanin (Roberts et al. 1991, 1990; Martinez and Chignell 1998; Kristensen et al. 1994). In the presence of any of these biomolecules, binding of the sensitizer to the biomolecule is indicated by a red shift in the absorption spectrum of the drug. For example, the Soret band of a porphyrin is shifted to the red in the presence of cytosol lens proteins (Figure 28.4) compared to the porphyrin alone, indicating binding of the porphyrin to the lens protein. Binding of a drug to an ocular tissue would increase its retention time in the eye and therefore the drug would be more likely to induce phototoxic damage (Roberts et al. 1991).

Finally, any reports of skin phototoxicity for a particular drug should provide a clear warning of potential ocular phototoxicity. Skin phototoxicity is more readily apparent than ocular phototoxicity, although it is induced by compounds with similar chemical features (Barratt 2004).

TABLE 28.1
Short Screen for Predicting Potential

1. Absorption spectrum	$\lambda > 295$ nm lens $\lambda > 400$ nm retina
2. Binding	Lens protein, DNA, Melanin
3. Chemical structure	Heterocyclic, tricyclic, porphyrin
4. Solubility	Amphiphilic/lipophilic
5. Skin phototoxicity	

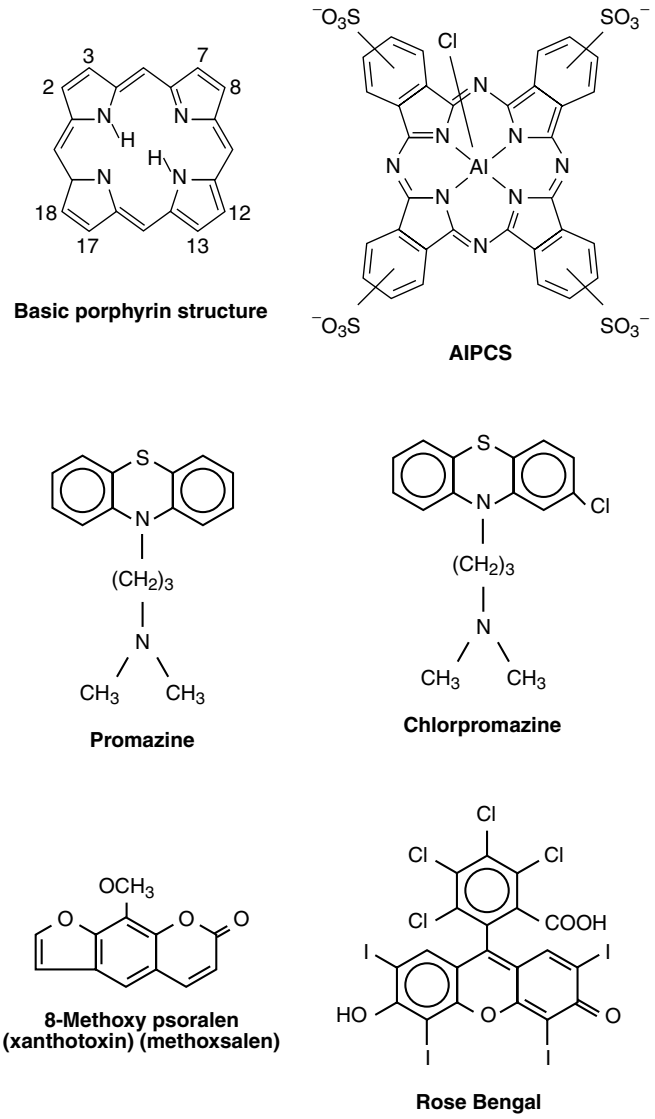


FIGURE 28.3 The chemical structures of photosensitizer—porphyrin, tricyclic, and heterocyclic ring systems.

28.5 ADDITIONAL TECHNIQUES

The simple screen presented earlier gives the first clear indication of whether or not a substance might be potentially

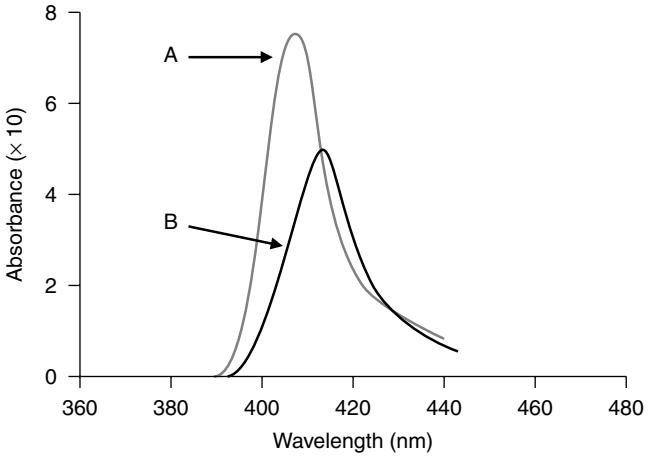


FIGURE 28.4 The Soret band of porphyrin (A); with the addition of lens protein there is a red shift (B) of this band, which is indicative of binding.

phototoxic to ocular tissues. It is a very valuable tool to screen out substances that *will not* be photosensitizers in the eye. Once it has been determined that a substance is a potential photosensitizer, additional *in vitro* and biophysical assays, which take into consideration the photochemical mechanisms of phototoxicity, are useful to get a more accurate assessment of potential phototoxicity.

28.6 PHOTOCHEMICAL MECHANISM OF PHOTOTOXICITY

The molecular mechanism involved in the phototoxic damage induced in the eye is through a photooxidation reaction. This reaction begins with the absorption of light by the sensitizer (drug, dye, herbal medication), which excites the compound to the singlet state (fluorescence) and then, through intersystem crossing, goes to the triplet state. It is generally the excited triplet state of the drug/dye that then proceeds either via a type I (free radical) or type II (singlet oxygen) mechanism to cause the eventual biological damage (Spikes 1998).

Photooxidation reaction

Drug + light →

singlet → triplet →

free radicals/reactive oxygen species →

ocular damage

28.7 BIOPHYSICAL STUDIES

In complex biological systems like the eye, photooxidation can occur by either a type I or a type II mechanism or by both concurrently. Additional information about the precise excited state intermediates produced and the efficiency of production (quantum yield) for a phototoxic reaction in the eye can be obtained by using several photophysical techniques (flash photolysis, luminescence, pulse radiolysis, electron spin resonance) (Broniec et al. 2005; Motten et al. 1999; Roberts et al. 2002, 2000; Roberts 2001; Bilski et al. 1998; Reszka et al. 1992; Gorman and Rodgers 1992). Determining the specific reactive intermediate(s) produced by a particuar sensitizer not only defines the mechanism of toxicity but can also later be used as a tool to prevent the damage. The techniques and the reactive species that are measured are summarized in Table 28.2.

TABLE 28.2
Biophysical Studies

Technique	Reactive Species
Laser flash photolysis	Triplet
Luminescence	Singlet oxygen, excited singlet, and triplet states
Pulse radiolysis	Radicals and oxyradicals
ESR	Radicals and oxyradicals

The extent of photooxidation is also influenced by the oxygen content of ocular tissues. Thus in addition to measuring excited-state intermediates, the measurement of the oxygen content of a particular component of the eye is useful. The cornea is highly oxygenated and its content can be measured by the single-chamber polarographic oxygen permeability measurement method (Weissman et al. 1990). The retina is supplied with oxygen by the blood so that different portions of retinal tissues have varying but high oxygen content. This may be measured, noninvasively and *in vivo*, with a scanning laser ophthalmoscope (Ashman et al. 2001). The aqueous and the lens have low oxygen content but it is sufficient for photooxidation to occur. Measuring the excited-state lifetime of phosphorescent dyes in the anterior chamber provides a useful method for determining oxygen concentration *in vivo*, without penetrating the eye (McLaren et al. 1998).

We have confirmed that photophysical studies correlate well with *in vivo* data (Roberts et al. 1991). For instance, TPPS, which binds to lens proteins, shows a long-lived triplet in the intact calf and human lens, and produces singlet oxygen efficiently; it also causes photooxidative damage *in vivo* in pigmented mice.

28.8 *IN VITRO* STUDIES

28.8.1 Location/Uptake of the Dye/Drug

For a drug, dye, or herbal medication to have a toxic effect, it must first be taken up into some compartment of the eye. The classical method for determining uptake into ocular tissues is *in vivo* radiolabeling. This method is time-consuming and expensive, although it is effective in determining which ocular tissues have accumulated the drug in question.

An alternative method to determine uptake of a drug into ocular tissues is ocular fluorometry (Taroni et al. 2003). After a dye or drug has absorbed light and is excited to the singlet state, it can decay to the ground state, accompanied by the emission of light (fluorescence). Since most

TABLE 28.3
***In Vitro* Studies**

Technique	Substrate
Fluorescence	Sensitizer uptake
Cell culture	DNA, RNA, protein synthesis
Comet assay	DNA cross-links
Enzyme assays	Antioxidant enzymes
Histology	Endothelial, epithelial, photoreceptor cell damage
Scan Tox™	Lens integrity
Gel electrophoresis	Protein changes
Amino acid analysis	
Mass spectrometry	Lipid changes
	Peptide maps
TLC	Lipid oxidation
High pressure liquid chromatography (HPLC)	Lipid peroxides
	DNA adducts
	Protein modification

photosensitizers are fluorescent, transmitted or reflective fluorescence provides accurate means of measuring uptake of a sensitizer into ocular tissue, a measurement that is simpler, less expensive, and less arduous than using radiolabeled materials. This technique may also be used noninvasively, *in vivo*, as for instance, in using a slit lamp to detect uptake of sensitizers into the human eye, or in using scanning laser ophthalmoscopes, or reflective fluorometry to determine the presence of endogenous and exogenous fluorescent materials in the lens or retina (Elsner et al. 2002; Cubeddu et al. 1999; Taroni et al. 2005; He et al. 2004; Sgarbossa et al. 2000) (Table 28.3).

28.8.2 Substrates of Photooxidative Damage

The targets of photooxidative reactions may be proteins, lipids, DNA, RNA, or cell membranes (Spikes 1998). *In vitro* tests can be designed to determine the specific site(s) of damage to the various ocular compartments (i.e., lens and retinal epithelial cells and photoreceptor cells) and the products of those reactions.

28.8.3 Complementary DNA Microarray Technology

Complementary DNA (cDNA) microarray technology has been developed to decode the complex genetic networks altered in response to environmental insults and disease (Wilson et al. 2002). This technique has been used by Andley to uncover the UV-A effects on human lens epithelial cells (Andley et al. 1994). This group (Andley et al. 2004) conducted a genome-wide screen of UV-A-induced changes in human lens epithelial gene expression. They found that a single dose of UV-A radiation (365 nm) modified genes linked with signal transduction, nucleic acid binding, and enzymes with a majority of genes repressed. This technique may now be applied to compare the effect of UVA alone to that of UVA with phototoxic agents.

28.8.3.1 Comet Assay

The comet assay is a very sensitive way of measuring low levels of DNA damage in individual cells (Singh et al. 1988). It is basically single-cell gel electrophoresis, which can measure oxidative-induced base damage, DNA–DNA/DNA–protein cross-linking, and both single-stranded and double-stranded breaks in DNA. This technique has been used to measure UV damage to both corneal epithelial cells (Choy et al. 2005) and retinal pigment epithelial cells (Roberts et al. 2002).

28.8.4 Scan Tox System—Focal Length Variability of the Lens

The Scan Tox system, which measures focal length variability, is a method for monitoring lens optical quality in culture conditions that mimic conditions inside the eye (Dovrat and Sivak 2005). The ocular lens is an ideal organ for long-term culture experiments because it has no direct blood supply and no connection to the nervous system; the Scan Tox system makes it possible to keep lenses for long-term studies of up to several weeks. The use of cultured lenses, mainly bovine, replaces the need for testing the effects of potentially damaging agents on live animals.

The optical monitoring apparatus uses a computer-operated scanning laser beam, a video camera system, and a video frame analyzer to record the focal length and transmittance of the cultured lens. The scanner is designed to measure the focal length at points across the diameter of the lens. The lens container permits the lens to be exposed to a vertical laser beam from below. The laser source projects its light onto a plain mirror, which is mounted at 45° on a carriage assembly. The mirror reflects the laser beam directly up through the test lens. The mirror carriage is connected to a positioning motor, which moves the laser beam across the lens. The camera sees the cross section of the beams and by examining the image at each position of the mirror, Scan Tox software is able to measure the quality of the lens by calculating the back vertex distance for each beam position. The cultured lenses continue to maintain their original refractive function.

When foreign substances are introduced to a cultured lens, the Scan Tox system measures the resulting optical response, providing a very sensitive means to follow early damage to the eye lens. It has recently been used to define the ocular toxicity of radiofrequency radiation (Dovrat et al. 2005) and the phototoxicity of hypericin, a phototoxic component of St. John's wort (Wahlman et al. 2003).

28.8.4.1 Gel Electrophoresis, Amino Acid Analysis

Gel electrophoresis has been used to monitor polymerization of ocular proteins (Kristensen et al. 1995; Roberts 1992; Roberts et al. 1985; Roberts 1984; Zigler et al. 1982). Photopolymerization is one of the most apparent changes in ocular protein induced by photosensitizing dyes and drugs.

Quantitative changes can be measured by scanning the gel and determining relative reaction rates. Specific amino acid modifications can be determined using amino acid analysis (Roberts 1984). Zhu and Crouch (1992) have illustrated the wide variety of classical protein analysis techniques (gel electrophoresis, amino acid analysis, sequencing, isoelectric point determination, western blot, ELISA) that can be used to investigate phototoxic damage induced by dyes and drugs.

28.8.4.2 Mass Spectrometry

Recent innovations in the field of mass spectrometry (liquid secondary ion mass spectrometry [LSIMS] and electrospray ionization [ESI]) have allowed for the identification of specific amino acid modifications within large proteins through molecular weight mapping. These techniques have been applied to determine the specific sites of photooxidative damage in corneal, lenticular, and retinal proteins (Schey et al. 2000; Roberts et al. 2001; Ablonczy et al. 2005).

These studies can serve as a model for defining damage from any potential phototoxic agent in the eye.

28.8.4.3 Thin Layer Chromatography

Thin layer chromatography is the method of choice for separating free fatty acids and phospholipids from lens (Fleschner and Cenedella 1997) and retinal (Organisciak et al. 1992) membranes. Thin layer chromatography/gas mass/mass spectrometry (TLC/GC/Mass Spec) may be used to measure lenticular or retinal lipid modifications (Handelman 2001). Specific lipids may be modified in the presence of photosensitizing agents and separated on TLC plates. The plates can then be scanned for quantitative analysis of these specific changes.

28.8.4.4 High Pressure Liquid Chromatography

HPLC is particularly effective at separating and identifying lipid peroxides from the retina (Akasaka et al. 1993). It has also been used to identify adducts formed between DNA nucleotides and phototoxic agents (Oroskar et al. 1994). HPLC has been used to assess the rates of photooxidation of lens proteins in the presence of a sensitizer; with this technique it is possible to determine which amino acid modifications have been induced within the protein, where they are located, and whether sensitizing drugs may have been bound to specific lens crystallines (McDermott et al. 1991).

28.8.4.5 Normalization for Photons Absorbed

Whatever the target tissue or extent of damage, the toxic effects of these dyes and drugs are the result of photochemical reactions. As such, their efficiency depends strongly on the number of photons absorbed by the sensitizer in the biological tissue. Therefore, to get an accurate comparison of the photosensitizing potency of various dyes and drugs with different structures and absorptive characteristics, it is essential to normalize for the number of photons absorbed by each drug in a particular system.

Normalization can be accomplished with a simple computer-generated mathematical formula (Roberts 2004), which takes into account the absorption spectrum of the drug, the output of the lamp source used in the experiments, and the optical properties of the eye. The total relative number of photons absorbed by a drug under particular experimental conditions is the area under the product curve:

$$\text{Photons absorbed} = I \times \text{AB} \times \lambda$$

where I is the intensity of the lamp at various wavelengths, adjusted for the transmission characteristics of the cornea or lens; AB is the absorbance of the dye/drug; and λ is the number of photons per energy unit at those wavelengths. The rate of photooxidative damage is then adjusted accordingly for each sensitizer.

28.8.4.6 Cell Culture/Whole Tissues

The first reported assay for phototoxicity in human cells (Roberts 1981) measured changes in macromolecular synthesis in the presence and absence of a light-activated drug. Other studies have assessed damage to corneal, lenticular, and retinal cells by measuring pump function, DNA cross-links, and enzyme activities both *in vitro* and *in situ* (Andley 2001; Roberts et al. 2002; Organisciak and Winkler 1994; Rao and Zigler 1992).

Photochemical Reactions in Tissues

Excited state	→	Intermediates	→ Target and damage
		singlet oxygen	proteins → polymers
triplet		superoxide	lipid → peroxides
		OH·, ROO·	DNA, RNA → cross-links

In vitro techniques determine the potential damage done to an ocular substrate, which gives information about the photoefficiency of a drug should it be taken up into the various compartments of the eye. Additional information about the site of potential damage can be predicted based on which ocular substrates (proteins, DNA, lipids) are modified.

28.8.4.7 Site of Damage

There are numerous locations subjected to phototoxic damage in the eye. The site of damage is determined by the penetration of the drug and the transmission of the appropriate wavelengths of light to that site.

28.8.4.7.1 Cornea

Corneal epithelial and endothelial cells may be easily damaged, leading to keratitis (Pitts et al. 1976; Hull et al. 1983). However, these cells have a very efficient repair mechanism and the damage is rarely permanent.

28.8.4.7.2 Lens

The epithelial cells of the lens, whose function is to control transport to the lens, have direct contact with the aqueous and are thus most vulnerable to phototoxic damage. Damage to these cells would readily compromise the viability of the lens (Andley 2001; Roberts 2004). The lens fiber membrane

can be photochemically harmed through damage to the lipids or the main intrinsic membrane protein (Roberts et al. 1985). Such damage leads to changes in the refractive index resulting in a loss of transparency (opacification) (Benedek 1971).

Phototoxic reactions can cause a modification of certain amino acids (histadine, tryptophan, cysteine) (Roberts 1984; McDermott et al. 1991) or a covalent attachment of sensitizer to cytosol lens proteins. In either case, the physical properties of the protein are changed, leading to aggregation and finally opacification (cataractogenesis). The covalently bound chromophore may now act as an endogenous sensitizer, producing prolonged sensitivity to light. Since there is little turnover of lens proteins, this damage is cumulative.

28.8.4.7.3 Retina

Phototoxic damage can occur in retinal pigment epithelial tissues, the choroid, and the rod outer segments, which contain the photoreceptors. If the damage is not extensive, there are repair mechanisms to allow for recovery of retinal tissues. However, extensive phototoxic damage to the retina can lead to permanent blindness (Organisciak and Winkler 1994; Ham et al. 1982; Glickman 2002).

28.8.4.8 *In Vivo* Testing

The screens described earlier will not totally eliminate the need for accurate *in vivo* experiments. However, the function of these studies is to limit the need for *in vivo* testing of large numbers of drugs for ocular phototoxicity. Those drugs found to be highly likely to produce phototoxic side effects in the eye should be tested further in animal studies to determine the exact site and extent of damage to be expected in humans.

With simple, inexpensive *in vitro* testing, compounds can be checked for phototoxicity at the developmental stage. It may be that a portion of the molecule can be modified to reduce phototoxicity while leaving the primary drug effect intact. This may reduce the necessity of future, more costly, drug recalls.

28.8.4.9 Protection

Even if a drug has the potential to produce phototoxic side effects in the eye, no damage will be done if the specific wavelengths of optical radiation absorbed by the drug are blocked from transmittance to the eye. This can be easily done with wraparound eyeglasses, which incorporate specific filters (Sliney 2001; Merriam 1996). Furthermore, nontoxic quenchers and scavengers could be given in conjunction with the phototoxic drug to negate its ocular side effects while still allowing for the primary effect of the drug (Roberts and Mathews-Roth 1993; Roberts et al. 1991, 2002; Roberts 1981 Busch et al. 1999; Wuet al. 2004).

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29 Water: Is It an Irritant?

Tsen-Fang Tsai

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An irritant is defined as any agent, physical or chemical, which is capable of producing cell damage. Everything can be an irritant if applied for sufficient time and in sufficient concentration. Water, being the most abundant element of the skin, is usually regarded as banal and gentle. However, the irritancy of water is beyond doubt. All nature evolves from the water. However, as man evolved from the water and became adapted to the earthy environment, the protection from water became one of the chief functions of the skin, which is the major protective organ of the human beings. Except in the fetus, protected by vernix caseosa, prolonged soaking in water is incompatible with human life.

Irritant contact dermatitis is the hallmark of an irritant reaction. It has been traditionally classified into an acute and chronic type. Strong irritants will induce a clinical reaction in a single application whereas with less potent irritants the response may be delayed and subclinical, requiring repeated or prolonged application (Hassing et al., 1982). However, not all irritant reactions manifest as dermatitis. Water, being an unconventional irritant, may irritate the skin in a way other than dermatitis. Fingertip dermatitis, or wear and tear dermatitis, is the best example of cumulative irritant reaction. In this condition, hands are chronically irritated by a variety of insults, especially water. The involved skin is hardened and fissured, but typical signs of dermatitis or inflammation such as erythema, swelling, or scaling are often lacking in the early stage. People who deal with wet work, such as hair dressers, hospital cleaners, cannery workers, bartenders, and hydrotherapists (Lazarov et al., 2005, p. 327), are especially at risk (Meding and Swanbeck, 1990). In rare conditions, water may also produce pruritus (Potasman et al., 1990), or pain (Shelley and Shelley, 1998) in susceptible patients. Substance P (Lotti et al., 1994, p. 232) and VIP (Misery et al., 2003, p. 195), respectively, have been implicated in their pathogenesis. Another water-induced condition is aquagenic urticaria (Medeiros, 1996), in which impurity and osmolarity of water may be important. Water as solvent for putative epidermal antigen has been proposed for its pathogenesis (Czarnetzku et al., 1986, p. 623).

Occlusive patch test is the gold standard for the study of contact dermatitis and the irritancy of water under occlusion has likewise attracted most clinical attention. Prolonged warm water immersion under occlusive shoes was considered to be the culprit of tropical-immersion-foot (Taplin et al., 1967). This is a condition of painful swollen feet first noticed in soldiers during the Vietnam war. Another condition is juvenile plantar dermatosis in which children, mostly atopic, present with dry, glazed, and fissured forefeet. Repeated wet-to-dry process in conjunction with friction was incriminated as the main cause.

Occlusive dressing has long been used as an effective adjuvant therapy for diverse conditions such as keloid (Sawada and Sone, 1992), periungual verrucae (Litt, 1978), and psoriasis (Broby-Johansen and Kristensen, 1989). Occlusion has been demonstrated to modify reactive events in Langerhans cells, and has profound effect on cytokine production (Wood et al., 1994). Occlusion can be achieved with either plastic dressing, silicone, or by water-soaked patches. Normal skin will show typical signs of inflammation such as vasodilation, perivenular lymphocytic infiltration, edema, mast cells degradation, and proliferation of fibroblasts after occlusion for up to two weeks (Kligman, 1996). Agner and Serup (1993) studied skin reactions after closed patch tests and six of twenty participants had a grade 1 clinical response to water after occlusion for 24 h. The irritation of water under occlusion can result from the water *per se* or from retention of sweat, which is far more irritative than the water *per se* (Hu, 1991). However, a state of anhidrosis will result after prolong occlusion (Papa, 1972; Sulzberger and Harris, 1972).

A normal water gradient is required for a healthy skin. The outermost layer of stratum corneum contains 10–30% water, while the viable epidermis contains roughly 70% water. In the stratum corneum, topically applied water exerts mechanical stress on individual corneocytes resulting in an alteration of barrier function. Treatment with distilled water results in swelling of stratum corneum cells and formation of massive water inclusions between adjacent cell layers. Corneocytes near the live-dead transition zone can swell nearly to double their thickness (Richter et al., 2004, p. 246). In the viable epidermis, the control of water passage is more complex. Water can slowly permeate the lipid bilayer by simple diffusion. In addition, some specialized cell membranes show higher water permeability. Water channel proteins, aquaporins, mediate the efficient movement of water

across the membrane. Skin bears abundant water channel aquaporin 3, important in the maintenance of cell volume (Matsuzaki et al., 2002, p. 85).

Water, as an irritant, exerts its damaging effect on the skin through different mechanisms. Skin occlusion will induce a change in the water gradient, and an adaptation of skin physiology ensures accordingly. The normal desquamation process is highly dependent on the water gradient of the stratum corneum. Increased water content of the stratum corneum will dilute the enzymes and change the pH value important for the corneodesmolysis (Watkinson et al., 2001). As a result, in macerated skin, the stratum corneum shows retentional hyperkeratosis and is shed in large sheets. Water may also inactivate type 1 transglutaminase and result in a special condition called self-healing collodian baby. Increased water content in the stratum corneum will also have a negative feedback response on the formation of natural moisturizing factors (NMFs) through the deactivation of keratohyalin granules degradation. Keratohyalin granules are known to be the main source of NMFs. The skin surface becomes excessive dry after the removal of occlusion. This drying effect of water is best demonstrated in wet packing for management of exudative lesions.

The importance of water as a primary irritant was demonstrated by Willis in 1973. Clinical and histological observations of skin occluded for 72–144 h revealed intense subacute dermatitis (Willis, 1973, p. 166). In 1997, Ramsing et al. have also induced experimental irritation by sodium lauryl sulfate in 21 healthy volunteers; one hand was exposed to water for 15 min twice daily for two weeks, while the other hand served as control. Water did not significantly influence transepidermal water loss, but caused a significant increase in skin blood flow, as evaluated by laser Doppler flowmetry. Clinical evaluation did not show any difference of dryness or scaling in this study (Ramsing and Agner, 1997). Without occlusion, the irritancy of water by itself is questionable in this model. However, it is impossible to clearly separate the effects of occlusion and water. The effect of occlusion must be conduction to the skin through water as a medium under physiologic condition. And even though erythema alone does not equate to irritancy, temperature stimulated erythema has been observed to augment pre-existing irritation (Loffler, 2001). Thus, water may also exert its irritancy through its other nonchemical nature. The temperature dependency of irritation has been well recognized (Berardesca et al., 1995, p. 83; Ohlenschlaeger et al., 1996). Besides, hydration changes the optics of the skin, and increases the penetration and absorption of the ultraviolet light. Photo bleaching of the melanin is also more prominent in dampened hairs and swimmers (Dubief, 1992; Basler et al., 2000, p. 299).

Persistent hydration of the skin surface also changes the ecological environment and supports the overgrowth of pathological organisms on the skin (Roth and James, 1989; Faergemann et al., 1983; Aly et al., 1978; Rajka et al., 1981). Diaper rashes and pitted keratolysis are the best examples. Dermatophytosis complex of the toewebs is likely affected. Occlusion alone may clear the periungual verrucae, and spread the mucosal type HPV, that is, condylomata acuminata, to the extragenital areas. Extraction of water soluble substances, or NMFs, from the skin is another mechanism. NMFs are a goup of water extractable substances, including sodium pyrrolidone carboxylic acid, sodium calcium lactate, amino acids, urea, and a sugar–protein complex. These substances can bind three to four times their own weight of water (Jacobi, 1959; Bank, 1952; Yamamura and Tezuka, 1989). The presence of water in the stratum corneum relies on an intercellular bilayer membrane that encloses the NMFs as in an envelope (Imokawa et al., 1991). Since water is the main plasticizing factor of the horny layer, the water content of the stratum corneum decreases when the NMFs are reduced, and superficial cracks might develop. The amino acid contents in senile skin are decreased (Jacobson et al., 1990). Frequent showering removes these water extractable substances and a delay in the replenishment of NMFs in aging skin may further aggravate this situation. It is for these reasons that frequent or prolonged bathing and showering, even without the use of soaps, is discouraged for the care of dry and senile skin (Hogstel, 1983).

Water may also interfere with electrolyte homeostasis and cause skin wrinkling. Water diffuses into the porous skin of the hands and soles via its many sweat ducts. Altered epidermal electrolyte homeostasis may cause changes in membrane stability of the surrounding dense network of nerve fibers and trigger increased vasomotor firing with subsequent vasoconstriction. Vasoconstriction, through loss of volume, leads to negative digit pulp pressure resulting in a downward pull on the overlying skin, which wrinkles as it is distorted. Impairment in this process may result in transient reactive papulotranslucent acrokeratoderma (English, 1996, p. 686), also called aquagenic keratoderma (Yan, 2001, p. 696) and aquagenic syringeal acrokeratoderma (MacCormack, 2001, p. 124). It is especially common in patients with cystic fibrosis, and has been reported to occur after amikacin (Katz, 2005, p. 621) or tobramycin treatment (Ludgate, 2006). Bedside immersion-wrinkling test is used as a test of autonomic digital nerve function, which is impaired in diabetes mellitus and trauma (Wilder-Smith, 2004, p. 125).

The importance of skin surface acidity was only unveiled recently after a long dispute (Schmid and Korting, 1995). This acidic milieu is vital for both the integrity of barrier function and for the regulation of skin flora (Rippke et al., 2002). The skin surface pH has also been found to be predictor for the development of irritant contact dermatitis (Wilhelm and Maibach, 1990). The irritancy of water can theoretically also result from its neutral pH of 7.0, which is alkaline compared to skin surface pH of between 4.2 and 6.0. The origin of this skin surface pH has remained enigmatic. A recent study implicates urocanic acid as the key factor in the maintenance of this acid mantle (Krien and Kermici, 2000). The neutralization capacity of lesional skin in hand eczema has been shown to be defective (Schieferstein and Krich-Klobil, 1982). The change in skin surface pH has been shown in atopic dermatitis, ichthyosis, diabetes mellitis, and patients on dialysis.

Water is a universal solvent. The trace elements in the thermal water are the corner stone of the alleged beneficial effect of crenotherapy. On the contrary, the hardness of water may sometimes contribute to the irritancy of water (Warren and Ertel, 1997). Hypotonicity of pure water, and change of water pressure gradient across the stratum corneum, which may trigger the release of cytokines, may also play a role in the irritancy of water. Specific osmotic sensitive receptor, such as TRPV4, may also be involved (Liedtke et al., 2000, p. 525). The same receptor may also be activated by heat (Guler et al., 2002, p. 6408), low pH, and citrate (Suzuki et al., 2003, p. 22664). Hydration of the stratum corneum also facilitates the penetration of foreign substances, and contributes to the development of allergic and irritant contact dermatitis. This is best exemplified in occupational contact dermatitis involving wet work (Meding and Swanbeck, 1990). Occlusive dressing therapy and wet wrapping therapy involve the same principle to enhance the therapeutic effects of topical corticosteroids (Sauer, 1977).

Water is the most important element of the human body. The control of water passage is a highly regulated, but poorly studied process. In the skin, it was previously considered to be a passive process controlled by the "dead" stratum corneum. But recent studies have revealed the importance of aquaporin, TRPV4, hyaluronic acid, and its receptor. To maintain this water homeostasis, a relatively dry and impermeable skin is highly desirable. Any change in this water gradient will bring about major changes in skin physiology. Water is a ubiquitous irritant, and exerts its irritancy through different mechanisms. The irritancy of water under occlusion has long been recognized. But even contact with pure water will produce physiologic changes of the skin, and these changes might be involved in some pathological processes. The irritancy of water is controlled by the quality and quantity of water as well as by individual susceptibility, including genetic predisposition and concomitant diseases, especially atopic dermatitis. Everything can be an irritant, including water.

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30 Sodium Lauryl Sulfate

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30.1 INTRODUCTION

Sodium lauryl sulfate (SLS) is an anionic surface active agent used as an emulsifier in many pharmaceutical vehicles, cosmetics, foaming dentifrices, and foods, and it is the sodium salt of lauryl sulfate that conforms to the formula: $CH_3(CH_2)10CH_2OSO_3Na$.[1] The action of SLS on surface tension is putatively the cause of its irritancy, and its great capacity for altering the stratum corneum (SC) makes it useful to enhance penetration of other substances in patch tests and in animal assays.

Kligman[2] found no sensitization to SLS was seen in hundred volunteers in which SLS was employed in provocative or prophetic patch-test procedures. There are isolated reports of contact sensitization to SLS.[3–5] Some important characteristics have been proposed for irritants used experimentally: no systemic toxicity, noncarcinogenic, not a sensitizer, chemically well defined, no extreme pH value, and not a cause of cosmetic inconveniences to exposed subjects.[6] SLS fulfills these criteria as a model irritant in the study of experimental irritant contact dermatitis.

30.2 APPLICATION METHODS

Many studies concerned with cutaneous irritation utilize a 24-h patch application. A 7-h patch[7] and 4-h patch[8] with high concentration of SLS have been developed. In real life, surfactant exposure is usually of short duration, open application, and cumulative. A single challenge of the skin with an irritant insult is a momentary reflection of the skin's susceptibility, which does not consider the cumulative effect of irritation or the repair mechanisms of the skin. Repetitive challenges allow for these effects. Assay methods similar to real usage situation such as repeated short duration chamber test,[9,10] repeated open application test,[11–14] plastic occlusion stress test (POST),[15,16] and soak or wash test[17,18] were developed.

A correlation coefficient of 0.63 between a single exposure and a 4-day repetitive exposure to patch testing with SLS was found.[19] With repeated open application of SLS for 5 days as well as a single 24-h patch test with SLS using small (8 mm) patch-test chambers, only the degree of skin damage caused by the repeated open test was found associated

with prior skin complaints.[20] Lammintausta et al.[11] observed the decrease in patch-test reactivity secondary to cumulative open SLS application using small (8 mm) patch-test chambers and suggested that the induced hyporeactivity might be one of the false negative diagnostic patch tests. There are two contrasting responses of cumulative SLS irritation; hyporeactivity may be noted if epidermal responses, including hyperkeratosis and dryness, were major reactions to irritant; whereas, if dermal reactions, such as erythema and edema, were major components, hyperreactivity may develop.[21] Recently, Heinemann et al.[22] observed decreased response during the third week of 0.5% SLS irritation and the increase of ceramide first three weeks after irritation, and they suggested that ceramide one seemed to play a key role as a protective mechanism against repeated irritation.

Tupker et al.[23] divided the studies on SLS into two categories with respect to aims. The first category, provocative testing, concerns studies in which SLS is used to induce a definite skin reaction in all individuals. Aims of the first category are to elucidate the mechanisms of skin irritation, to predict the irritant potency of different detergents, to study the time course after irritation, to compare the sensitivity of different noninvasive methods, to compare the efficacy of different moisturizers, barrier creams, or corticosteroids in preventing or healing skin irritation. The second category, susceptibility evaluation, concerns studies aimed to predict the irritant susceptibility of individuals, and investigate individual and environmental factors determining this susceptibility.

There are some variations in skin responses to identical patch tests and standardization of patch-test procedure is necessary to minimize the variations in patch-test responses. Tupker et al.[23] suggested the guidelines on SLS exposure tests.

30.2.1 Purity and Carbon Length of SLS

There were significant differences in the irritant potential *in vivo* for different qualities of SLS, and there were cases in which some of the C12 chains had been substituted by longer and less-irritating carbon chains.[24] The presence of C12 chains of SLS is known to elicit a maximum irritant reaction[25–28] suggested that only SLS qualities of high purity (>99%) should be used for irritant patch testing and that the quality and the purity of SLS should be stated.

30.2.2 Quantity and Concentration of Test Solution

Quantity of test solution is important and larger quantities of test solution give more intense skin reactions, although the concentration of the irritant is kept constant,[29,30] and Agner[31] suggested that the Duhring chamber, the 12-mm Finn chamber, or even large chambers having bigger test areas are more effective in eliciting a response. Mikulowska and Andersson[32] observed that the effects of 8-mm chambers could result in increased, unchanged, or decreased Langerhans cells (LC) numbers, while 12-mm chambers always produced decrease in LC numbers. Lee et al.[33] also compared the effect of chamber size on SLS irritation on the volar forearm using three different sizes (8, 12, and 18 mm) of Finn chambers. The increase in visual score and transepidermal water loss (TEWL) at the patch-tested sites with large (12 mm) Finn chamber was greater than that with the small (8 mm) Finn chamber. However, there were no significant differences between large and extralarge (18 mm) Finn chambers.

Aramaki et al.[34] studied the interrelationship between SLS concentration and duration of exposure in irritant skin reaction. The influence of SLS concentration and duration of exposure was demonstrated with a standardized coefficient value β. For TEWL, the β value of the SLS concentration was 1.5-fold higher than that found for the exposure time. For the laser Doppler flowmetry (LD), the β value of concentration was 2.5-fold higher than that of exposure time. And they suggested that the skin reaction to SLS could be calculated by the following formulae: $\Delta\text{TEWL} = 14.36 \times \text{concentration} + 0.82 \times \text{duration (hours)} - 5.12$, and $\text{LD} = 30.81 \times \text{concentration} + 1.09 \times \text{duration} + 2.49$. This estimation is only valid for a patch application of $\leq$24 h.

Brasch et al.[35] have analyzed the synchronous reproducibility of patch tests with various concentrations of SLS aqueous solution (0.0625, 0.125, 0.25, 0.5, and 1.0%) using large Finn chamber, and they suggested that 1.0% SLS aqueous solution is appropriate for an irritant patch test as a positive control. Contamination with bacteria was found in the SLS solutions of lower concentrations resulting in decreased concentration of SLS, and the storage of SLS solutions of very low concentrations should be at low temperature and preferably in sterile vials.[36]

30.2.3 Evaporation and Temperature of Test Solution

The penetration of SLS through the skin barrier is significantly increased by the increase of the temperature of test solution.[37] Berardesca et al.[38] reported significantly different skin responses to the temperature of test solution (4, 20, and 40°C). Skin damage was higher in sites treated with warmer temperatures, and there was a highly significant correlation between irritation and temperature of test solution. Ohlenschlaeger et al.[39] also demonstrated increased irritation on the application site of warmer solution using repeated immersions in an SLS solution at 20 and 40°C. Transition from a packed gel state to a more fluid crystalline state in SC lipids occurs at temperatures between 38 and 40°C, and the fluidity of SC is important in the percutaneous penetration process as an explanation of increased irritancy at higher temperatures.[38] The evaporation rate of aqueous solutions from Finn chambers was reported as 1 mg/3 min.[40] Evaporation from the patch before application inhibits the inflammatory response, even though the relative concentration of the irritant is increased by the evaporation process.[41] This inhibition of skin irritation could be the result of decreased amount or lowered temperature owing to evaporation of test solution.

30.2.4 Time of Evaluation

When noninvasive measurements of the skin response are made, the interval between removal of the patch and the measurements should allow for a period of increased evaporation following occlusion. Equalization of water diffusion between the SC and the ambient air is settled after 20 min of patch removal.[42] For measurements of TEWL, in most papers, the interval was reported to be 30 min.[43–45] The time course of TEWL after SLS patch testing demonstrated significant reduction in TEWL values from 30 to 60 min after removal of the patch, but not from 60 to 180 min,[46] and they suggested that evaluation of irritant patch-test reactions by measurement of TEWL can naturally be made at any time after removal of the patches, as long as the time period is precisely accounted for. Others have argued that a minimum waiting period of 2 or 3 h should be allowed for evaporation of excessive water due to occlusion.[19,47] Recently, Aramaki et al.[34] suggested that TEWL measurement performed 30 min after patch removal is too early and measurement 24 h after patch removal should be done for practical reasons.

30.2.5 Guidelines on SLS Exposure Methods

High-purity (99%) SLS must be used in any study, dissolved water in occlusive and open testing, while tap water may be acceptable in immersion testing. Standard-sized occlusion chambers with filter paper disks corresponding to large (12 mm, 60 μL) and extralarge (18 mm, 200 μL) Finn chamber are recommended. The extralarge Finn chambers are recommended for repeated applications. For open exposures, 20 mm diameter plastic ring is advised. The volume of the solutions must be such that the total exposure area is covered (800 μL). Chambers should be applied to the skin immediately, i.e., within 1 min after preparation with the test solution. TEWL measurement should be performed a minimum of 1 h after removal of test chambers. European Society of Contact Dermatitis (ESCD) proposed new guidelines in terms of purposes and methods of SLS exposure tests (Table 30.1).[23]

30.3 BIOLOGIC ENDPOINTS

30.3.1 Clinical Appearance of SLS Reaction

Erythema, infiltration, superficial erosion can be seen during acute reaction to SLS. With higher concentrations vesicular and pustular reactions may be seen. During healing of acute reactions, scaling and fissuring will take over. The same appearance of erythema, scaling, and fissuring is seen during repeated application of SLS. The soap effect consisting of fine wrinkled surface or chapping is not commonly seen in SLS patch-test reaction.[23] Most recently reported literatures have used the modified visual scoring system of Frosch and Kligman[9] to evaluate clinical skin reaction to SLS. Tupker et al.[23] developed the guideline concerning about the visual scoring schemes for the acute and cumulative reactions to SLS (Tables 30.2 and 30.3).

TABLE 30.1
ESCD Guidelines on SLS Exposure Tests with TEWL Measurement

	Susceptibility Evaluation		Provocative Testing	
	Acute	Cumulative	Acute	Cumulative
One-time occlusion test				
Application time	24 h	Not	24 h	Not
Mode of application	Chamber 12 mm	applicable	Chamber 12 mm	applicable
SLS w/v%	0.5%		2%	
Repeated occlusion test				
Application time	Not	2 h 1 × daily	Not	2 h 1 × daily
Application period	applicable	3 weeks[a]	applicable	3 weeks[a]
Mode of application		Chamber 18 mm		Chamber 18 mm
SLS w/v%		0.25%		1%
Open test				
Application time	60 min 2 × daily	10 min 1 × daily	Not possible[a]	10 min 1 × daily
Application period	1 day	3 weeks[a]		3 weeks[a]
Mode of application	20 mm guard ring	20 mm guard ring		20 mm guard ring
SLS w/v%	10%	1%		2%
Immersion test[b]				
Immersion time	30 min 2 × daily	10 min 2 × daily	30 min 2 × daily	10 min 1 × daily
Application period	1 day	3 weeks[a]	1 day	3 weeks[a]
Mode of application	Forearm immersion	Forearm immersion	Forearm immersion	Forearm immersion
SLS w/v%	0.5%	0.5%	2%	2%

Source: Tupker, R.A. et al., *Contact Dermatitis*, 37, 53, 1997.

[a] One week is 5 application days.

[b] Water temperature, 35°C.

TABLE 30.2
ESCD Guideline on Clinical Scoring of Acute SLS Irritant Reactions

Score	Qualification	Description
0	Negative	No reaction
1/2	Doubtful	Very weak erythema or minute scaling
1	Weak	Weak erythema, slight edema, slight scaling, and slight roughness
2	Moderate	Moderate degree of erythema, edema, scaling, roughness, erosions, vesicles, bullae, crusting, and fissuring
3	Strong	Marked degree of erythema, edema, scaling, roughness, erosions, vesicles, bullae, crusting, and fissuring
4	Very strong/caustic	As 3, with necrotic areas

Source: Tupker, R.A. et al., *Contact Dermatitis*, 37, 53, 1997.
Note: Reading 25–96 h after one-time exposure.

TABLE 30.3
ESCD Guideline on Clinical Scoring of Subacute/Cumulative SLS Irritant Reactions

Score	Qualification	Description
0	Negative	No reaction
½	Doubtful	Very weak erythema or shiny surface[a]
1	Weak	Weak erythema, diffuse or spotty, slight scaling, and slight roughness[b]
2	Moderate	Moderate degree of erythema, scaling, roughness, and weak edema and fine fissures
3	Strong	Marked degree of erythema, scaling, roughness, edema, fissures and presence of papules and erosions, and vesicles
4	Very strong/caustic	As 3, with necrotic areas

Source: Tupker, R.A. et al., *Contact Dermatitis*, 37, 53, 1997.

[a] The term shiny surface is used for those minimal reactions that can only be discerned when evaluated in skimming light as a "shiny area."

[b] The term roughness is used for reactions that can be felt as rough or dry, sometimes preceeded or followed by visible changes of the surface contour, in contrast to "scaling," which is accompanied by visible small flakes.

30.3.2 Pathogenesis of SLS Reaction

The histopathologic changes induced by SLS depend on various factors including concentration, mode of application, and time of evaluation. Acute reaction to SLS application in epidermis can include hyperkeratosis, parakeratosis, spongiosis, intracellular edema, hydropic degeneration of basal cells, and necrosis.[48–51] In dermis, there were variable degrees of inflammatory cell infiltration, edema, and collagen degeneration. T lymphocytes are the predominant infiltrating cells and CD4(+) cells outnumbered the CD8(+) cells.[52–56] The histological changes to cumulative SLS irritation were similar as in acute irritation, but repetitive mild irritation may evoke epidermal hyperplasia with minimal inflammatory infiltration.[48]

Many surfactants including SLS disrupt the skin barrier function resulting in increased TEWL,[57,58] and increased blood flow, clinically visible as erythema.[59] Leveque et al.[60] suggested that an increase in TEWL did not necessarily imply the alteration of SC and SLS-induced dry skin could hardly be interpreted in terms of lipid removal.[61] A disruption of the secondary and tertiary structure of keratin proteins may expose new water-binding sites resulting in SC hydration, and the most likely explanation of SLS-induced increase in TEWL lied in the hyperhydration of SC and a possible disorganization of lipid bilayers.[27] Forslind[62] proposed a domain mosaic model of skin barrier. SC lipids are not randomly distributed, but are organized in domains. Lipids with very long chain lengths are segregated in gel, impermeable to water, and separated by grain borders populated by lipids with short chain lengths, which are in fluid phase, permeable to water. Surfactants including SLS infiltrate the fluid phase permeable to water increasing the width of grain borders, and increase TEWL.

30.3.3 Noninvasive Bioengineering Techniques Assessing SLS Reaction

Several noninvasive bioengineering methods to quantify and to obtain information which is not detectable clinically have developed in recent decades (Table 30.4).[63] Measurement of TEWL as a technique to evaluate skin barrier function is widely used[64,65] and a positive dose–response relationship for skin response to SLS as measured by TEWL has been demonstrated.[66] When attempting to quantify irritant patch-test reactions by electrical conductance measurement, the intraindividual variation in the results was so high that the method was found unhelpful for this purpose.[67] A positive relationship was found between dose of SLS and blood flow values recorded by LD.[66,68] However, wide fluctuations in laser Doppler blood flow values in response to SLS patches were found due to spotty erythema.[44] The skin color is expressed in a three-dimensional coordinate system: a* (from green to red), b* (from blue to yellow), and L* (from black to white) values.[69] Color a* coordinates have been demonstrated to correlate well with visual scoring of erythema in inflammatory reactions caused by soap or SLS.[65,70,71] Ultrasound examination has the advantage that no preconditioning of the subjects is necessary before measurement. Ultrasound A-scan has been found suitable for quantification of patch-test reactions[72,73] and also a promising method for quantification of SLS-induced inflammatory response, being

TABLE 30.4
Noninvasive Bioengineering Techniques Used in the Evaluation of Cutaneous Irritation

Technique	Measured Skin Function	Informed Obtained
Evaporimeter	Transepidermal water loss	Positive dose–response relationship for skin response to SLS Most sensitive method for SLS-induced irritation
Laser-Doppler flowmeter	Blood flow	Positive relationship between applied dose of SLS and blood flows. Wide fluctuations in response to SLS due to spotty erythema
Ultrasound	Skin thickness	No preconditioning is necessary. Good relation to SLS concentrations, but minimal correlation with erythema or epidermal damage
Impedance, conductance, capacitance	Skin hydration	Correlation with epidermal damage, but intraindividual variation is so high, this method is unhelpful
Colorimeter	Skin colors	Positive correlation between changes in the a* color coordinates and doses of SLS, but not with epidermal damage

Source: Lee, C.H. and Maibach, H.I., *Contact Dermatitis*, 33, 1, 1995.

consistently more sensitive than measurement of skin color,[66] and Seidenari and di Nardo[74] demonstrated that B-scanning evaluation showed a good correlation with TEWL values in assessing superficial skin damage induced by SLS.

In a comparison among evaporimetry, LD, ultrasound A-scan, and measurement of skin color, evaporimetry was found to be the best suited method for evaluation of SLS-induced skin damage.[65,67] Lee et al.[75] also observed that measurement of erythema index using Dermaspectrometer was less sensitive than TEWL measurement. However, Wilhelm et al.[65] suggested that although TEWL measurements may be an accurate and sensitive method in evaluating skin irritation, color reflectance measurements may be a helpful complimentary tool for the clinician, because of its convenience. Serup[76] suggested that measurement of TEWL is sensitive and useful in the study of corrosive irritants, such as SLS, especially in the induction phase of irritant reaction, but does not have direct clinical relevance, and the results need to be backed up with other relevant measures. Fluhr et al.[77] suggested that, regarding the time-dependent effect, a positive discrimination was seen for TEWL, measuring the barrier function, and the perfusion parameter LD. The discriminatory ability of TEWL was superior to that of LD. However, when evaluating SLS patch testing by bioengineering methods, TEWL measurement appears more suitable to evaluate skin reaction to SLS concentration $<1.0\%$, whereas LD is more appropriate to evaluate pronounced skin reaction (SLS concentration $\geq 1\%$).[78]

Tupker et al.[79] found that the time course of TEWL after a 24-h SLS patch test varied between different subjects. Using SLS in varying concentrations, Serup and Staberg[73] found a delayed response only for reactions clinically scored as 1+, but not for more intense reactions, indicating that the kinetics of the response may depend on the severity of the reaction.[78,]

30.3.4 Recovery of SLS Reaction

Wilhelm et al.[80] studied the skin function during healing phase after single 24-h patch application of 0.5% SLS solution. Erythema was most increased directly after patch removal with a slow gradual decrease, but not completely resolved even 18 days after treatment. SC hydration evaluated by capacitance measurements did not return to baseline values before 17 days after surfactant exposure. The repair of the SC barrier function as indicated by TEWL measurements was completed 14 days after exposure. Freeman and Maibach[44] described augmented irritant response to repeated application of 2% SLS solution on the clinically improved irritant contact dermatitis site, and suggested that although skin may appear to be morphologically normal, it may not be functionally normal. Lee et al.[21] suggested that complete recovery of skin function after acute reaction induced by 1% SLS solution was achieved approximately 4 weeks later. Choi et al.[81] demonstrated that skin reactivity of chronically irritated sites with SLS solution showed hyperreactivity compared to normal skin even 10 weeks after chronic irritation, and suggested that chronically irritated skin required a longer recovery time than acutely irritated skin.

30.3.5 Comparison of SLS Reaction with Noncorrosive Irritants

Irritants could be divided into two types: corrosive and noncorrosive irritants.[76] Corrosive irritants induce impairment of skin barrier function even when in provoked weak or subclinical reaction. Corrosive irritants have shown linear dose–response curve. However, noncorrosive irritants that cause low degree of irritation do not induce barrier disruption, and noncorrosive irritants may show linear dose–response at lower concentrations and have a tendency to make a plateau at higher concentrations. SLS has been considered as the typical corrosive irritant, and nonanoic acid (NAA) is an example of noncorrosive irritant. There are many reports comparing the skin responses between SLS and NAA; these are clinical morphology, histopathological changes, and changes in skin function measured by noninvasive bioengineering techniques.

Reiche et al.[82] observed the clinical morphology of SLS and NAA patch-site reactions and showed that erythema decreased with time for all concentrations of NAA and at higher concentrations of SLS. Surface changes increased with time for SLS patch sites and at higher concentrations of NAA. Lindberg et al.[83] studied the differential effects of SLS and NAA on the expression of CD1a and intercellular

adhesion molecule 1 (ICAM-1) in human epidermis. ICAM-1 reactivity could not be detected in epidermis on the site of 20 and 80% of NAA solution, and there was a decrease in CD1a+ cells after 80% NAA application. However, SLS induced ICAM-1 expression on keratinocytes, and the effects on the number of CD1a+ cells were minimal. Forsey et al.[84] compared the effects of NAA and SLS on the LCs and keratinocytes of clinically normal skin in patients with chronic irritant contact dermatitis. SLS induced keratinocyte proliferation after 48 h of exposure; however, NAA decreased keratinocyte proliferation after 24 h of exposure, but this returned to basal levels after 48 h. SLS induced keratinocyte apoptosis after 24 and 48 h of exposure; however, NAA induced epidermal cell apoptosis after only 6 h of exposure. SLS had no effect on LC number, and no CD1a+ apoptotic cells were seen after exposure to SLS. NAA dramatically decreased LC number after 24 and 48 h of exposure, which was accompanied by basal redistribution. Most significantly, NAA induced apoptosis in over half of LCs present after 24 and 48 h of exposure. Boxman et al.[85] observed immunoreactive HSP27 in the upper cell layers of the epidermis after exposure to the higher (2%) concentration of SLS. However, HSP27 nuclear immunoreactivity was observed in the skin exposed to the lowest concentration of NAA tested (2.5%).

Seidenari[86] compared the irritant reactions induced by NAA and SLS using 20-MHz B-scan. A clear decrease in flexibility of the epidermis echo at 24 h was visible at SLS patch-test sites, whereas at patch-test sites with NAA, there was a trend toward an increase in values of hyperreflecting pixels. Fullerton et al.[87] studied the skin irritation typing and grading using laser Doppler perfusion imaging. For SLS, both mean perfusion and area were found to increase from day 2 to day 3. The values decreased on day 5. The NAA reactions had a more rapid onset, peaked at 24 h (day 2) and then gradually declined at 48 and 96 h. We applied the SLS and NAA solutions on the volar forearm skin for 24 h and measured TEWL values and erythema indices to compare the different features of irritant reactions between corrosive and noncorrosive irritation. In our study of TEWL measurements, SLS solutions caused higher TEWL values than NAA, and the slope of SLS curve was steeper than that of NAA curve in relation to the concentration of SLS and NAA solutions. There was a tendency for the TEWL values to make a plateau at the higher NAA concentrations. However, both SLS and NAA solutions showed very similar pattern of erythema indices. In the study of the time course of TEWL values and erythema indices, TEWL returned to baseline values after 3 weeks in areas patch tested with 50% NAA. But TEWL values did not recover baseline values until 3 weeks in the corresponding areas tested with 5% SLS. However, erythema index curve of 5% SLS and 50% NAA showed quite similar pattern.[88] Benzalkonium chloride (BKC), another typical noncorrosive irritant, showed much less damage to the skin barrier function compared to the concentration of SLS, while they showed a similar degree of erythema. The slope of BKC was between those of SLS curve and NAA curve in relation to the concentration of SLS, NAA, and BKC solutions.[89]

TABLE 30.5
Host-Related Factors in Cutaneous Irritation

Age
Sex
Anatomic region
Race and skin color
Skin hydration
Sensitive skin
Hyperirritable skin
Skin disease (atopic dermatitis, hand eczema, seborrheic dermatitis)

30.4 HOST-RELATED FACTORS

There are many host-related factors in cutaneous irritation: those that are considered as skin disease and those that represent variations from normal skin predisposed to irritation (Table 30.5).

30.4.1 Age

Increased susceptibility to SLS in young females compared to elderly females was reported, when assessed by visual scoring and TEWL, and the increase in TEWL values was found to be more persistent in the older group.[90,91] These findings imply less reaction to an irritant stimulus but a prolonged healing period in older people. There is no significant influence on skin susceptibility between the 18 and 50 years of age,[92] but significantly reduced irritant reactivity in older more than 55 years age group compared to various younger age groups.[93]

30.4.2 Sex

Hand eczema occurs more frequently among women than men. However, many investigators have found no sex correlation in skin susceptibility.[45,94–96] Reactivity to SLS at day 1 increased in the menstrual cycle compared to day 9–11, when tested on opposite arms in healthy women.[97] Since no cyclical variation was found in baseline TEWL, the increased reactivity of the skin at day 1 in the menstrual cycle probably reflects an increased inflammatory reactivity, rather than changes in the barrier function. Recently, Robinson[98] reported that the male subjects responded more rapidly, and there was a significant increase in response of the male subjects compared to female subjects.

30.4.3 Anatomic Region

Variation in skin responses within the same individual to identical irritant patch tests may be considerable. Van der Valk and Maibach[99] studied the differences in sensitivity of volar surface of the forearm to SLS and demonstrated that the potential for irritation increases from the wrist to the cubital fossa, and Panisset et al.[100] showed that TEWL values next to the wrist were found greater than on the other sites of volar

forearm. Cua et al.[90] reported that the thigh had the highest reactivity and the palm the lowest. Henry et al.[101] studied the regional variability to 1% SLS using corneosurfametry bioassay and found that the dorsal hand and volar forearm were the least reactive, the neck, forehead, back, and dorsal foot the most reactive sites. Dahl et al.[102] found that, for simultaneous Al-patch testing with SLS, the corresponding sites on the right and the left sides were scored identically in only 53% of cases. Using large Finn chambers (12 mm), 84% of SLS patches showed identical visual score when tested simultaneously on right and left arms.[67] Rogiers[103] suggested that measurement of TEWL should be carried out on identical anatomic sites for all subjects involved, and the volar forearm is a good measurement site and corresponding places on the right and left forearms exhibit the same TEWL.

30.4.4 Race and Skin Color

Bjornberg et al.[104] reported that fair skin and blue eyes showed the high intensity of the inflammatory response to a mechanical irritant. When skin color was assessed by a tristimulus colorimeter, an association between light reflection (L*) from the skin surface and susceptibility to SLS was found.[92] By determination of minimal erythema dose (MED) in Caucasians, the cutaneous sensitivity to UV light and to seven different chemical irritants was found to correlate positively, while skin phototype based on complexion and history of sunburn proved less reliable.[105] McFadden et al.[106] found no significant differences in irritation thresholds to SLS among six skin phototypes. In contrast to these reports, an inclination to increased susceptibility to SLS in black and Hispanic skin types as compared to white skin types was found when evaluated by measurement of TEWL.[43,64] There were more complex reports concerning the SLS susceptibility between Caucasian and Asian population. There was an increased cumulative irritation response in Japanese subjects versus Caucasian to various chemicals.[107] Foy et al.[108] demonstrated a greater acute irritant responses in Japanese women compared to Caucasian; however, cumulative irritation did not show significant increase in Japanese compared to Caucasian. Chinese displayed similar response profile in acute irritation test; however, they showed a slower and less-severe response in the cumulative irritation test compared to Caucasian or Japanese subjects.[98] Robinson et al.[109] failed to find significant differences in skin reactivity to SLS between Caucasians and Asians. However, there was a consistent trend toward increased reactivity, i.e., reduced time to respond, observed in the Asian versus Caucasian subjects.[93] Tanning may influence the susceptibility to irritants. A diminished reaction to SLS after UVB exposure was reported.[110]

30.4.5 Skin Hydration

In repetitive exposure to SLS, higher susceptibility was reported in dry skin than in clinically normal skin in eczematous subjects and controls.[79] Comparing winter and summer skin, decreased skin hydration was found in winter, when a higher reactivity to SLS was also found.[24] Low outdoor temperature and low relative humidity in the winter lead to decreased ability of the SC to retain water.[111] Thus, these studies indicate that a decreased hydration state of the skin may be associated with impaired barrier function and increased skin susceptibility. In contrast, Lammintausta et al.[112] found no relationship between clinically dry skin and the response to repeated SLS exposure.

30.4.6 Sensitive Skin

Frosch and Kligman[113] reported a significant correlation between the skin response to particular irritants in healthy volunteers and patients with skin diseases. Murahata et al.[114] suggested a relationship between skin susceptibility to detergents and high baseline TEWL, and a highly significant correlation between baseline TEWL and TEWL after a single or repeated exposure to SLS was reported.[19,96,97] However, other studies reported an absent or poor correlation between baseline TEWL and TEWL after SLS exposure.[43,44,115,116]

Sensitive skin is a skin type having higher reactivity than normal skin and developing exaggerated reactions when exposed to external factors.[117] The stinging test using lactic acid has been widely used for the selection of sensitive skin. However, this test is based on self-perceived assessment and lacks objectivity. Seidenari et al.[118] demonstrated a decrease of baseline capacitance values indicating the tendency to barrier impairment, and they suggested that dehydration can represent a basis for subjective sensations after exposure to water and soap. Lammintausta et al.[104] demonstrated reactivity to a 24-h SLS patch test using LD in stingers compared with nonstingers. Simion et al.[119] also showed the correlations between self-perceived sensory responses to cleansing products and TEWL and colorimeter a* values in stingers. However, other studies did not show correlation between self-assessed skin sensitivity or skin reactivity to chemosensory stimuli and skin reaction to SLS irritation.[93,109,120] Recently, we performed lactic acid sting test, dimethyl sulfoxide (DMSO), and SLS patch tests in 55 Koreans. There were no significant differences in the skin responses of lactic acid sting test, DMSO, and SLS patch tests between sensitive and nonsensitive skin.[121]

Loeffler et al.[122] propose the new classification of skin irritancy. People with sensitive skin are only individuals who stated their skin as sensitive. There is no possibility to prove the statement with objective methods. If individuals react repeatedly to a skin test with sensation induced by chemical irritants such as lactic acid, they are identified as a stinger. If individuals do have an increased skin susceptibility to irritation caused by chemical irritants, which objectively be measured using bioengineering methods, they are identified as individuals with an irritable skin.

30.4.7 Irritable or Hyperirritable Skin (Excited Skin Syndrome)

Mitchell[123] introduced the term *angry back* to describe the phenomenon of a single strong positive patch-test reaction

creating a back, which is hyperreactive to other patch-test applications. The excited skin syndrome was illustrated experimentally in guinea pigs, and increased susceptibility to an ointment containing 1% SLS was observed in animals stressed by inflammatory reactions in the neck area.[124] Bruynzeel et al.[125] attempted to use SLS patches as markers of hyperirritability. Agner[126] observed no increased skin reactivity to SLS in patients with chronic or healed eczema compared to controls, while hand eczema patients with acute eczema showed increased skin reactivity to SLS compared to controls. Shahidullah et al.[127] reported increased TEWL values in the clinically normal skin of patients with eczema. But there was no significant difference in baseline TEWL values between patients with eczema and in controls.[126,128]

30.4.8 Skin Diseases (Atopic Dermatitis, Hand Eczema, Seborrheic Dermatitis)

There is a marked abnormality in barrier function in the skin of patients with atopic dermatitis (AD), and high levels of sphingomyelin deacylase were demonstrated in the lesional and nonlesional skin of patients with AD leading to decrease of ceramide and abnormality in barrier function.[129] Di Nardo et al.[130] suggested that SC ceramide content may determine a proclivity to SLS-induced irritant contact dermatitis. There are many reports of increased baseline TEWL in clinically normal skin of patients with AD.[79,131–135] Agner[132] showed that the response to SLS was statistically significantly increased in atopics compared to controls, when evaluated by visual scoring and skin thickness, but not TEWL. Nassif et al.[134] suggested that AD patients, as well as those with a history of allergic rhinitis, had lower irritant threshold than controls. It has also been demonstrated that a significantly greater response to SLS,[79,126,134,136] as well as a tendency to increased skin susceptibility, is related to the degree of severity of the dermatitis.[137] There were no significant differences in TEWL between individuals who were classified as atopic but without active dermatitis, individuals with rhinoconjunctivitis or atopic asthma and healthy controls, either at the basal or at the post-SLS measurement. Enhanced skin susceptibility is only present in individuals with active dermatitis.[138] Basketter et al.[139] also could not find significant differences in skin reactions to SLS in the normal skin of AD compared to control group.

Baseline TEWL values in patients with localized, inactive, or healed eczema were not significantly higher than in controls.[15,126] Agner[126] observed no increased skin reactivity to SLS in patients with chronic or healed eczema compared to controls, while hand eczema patients with acute eczema showed increased skin reactivity to SLS compared to controls.

There were several reports that patients with seborrheic dermatitis showed could be easily irritated to some chemicals including SLS.[136,140] Tollesson and Frithz[141] observed increased TEWL values and abnormality in essential fatty acids in infantile seborrheic dermatitis, and they normalized TEWL values by applying the borage oil containing gamma-linoleic acid.

30.5 CONCLUSION

It is clear that SLS data do not provide a unanimous opinion on all points. Yet, the preponderance of the observations suggest that we are beginning to understand some of the parameters, such as purity, dose, patch, anatomic site, single versus multiple application, occluded versus open application, that influence diverse response of the skin irritation.

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31 Factors Influencing Applied Amounts of Topical Preparations

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The effect of any medication depends on the applied dose. In case of topical medications, the applied amount is often uncertain and variable. The importance of proper dosage of topical medications in dermatology was seldom discussed, but accurate dosage of topical agents (e.g., calcipotriol) was demonstrated to provide better efficacy [1]. Additionally, to make any conclusion about the effectiveness of a topical preparation, it is preferable to have the amount of a topical preparation known and well controlled.

Unfortunately, only limited scientific data are available on the variability of amount of topical preparations applied to the skin of the patients in different conditions. Some studies concerned not pharmaceutical preparations, but sunscreens; others dealt with application of barrier creams. Nevertheless, with certain limitations, the data of sunscreen and barrier cream studies might be generalized to all topical preparations, including topical pharmaceuticals.

Our aim was to collect and analyze the available information on the factors influencing the amount of various topical agents applied to skin.

The analysis of the data demonstrated that the amount of applied topical agents depended on several factors, which can be divided into two main groups: (1) preparation-dependent, and (2) patient-dependent.

31.1 PREPARATION-DEPENDENT FACTORS

Preparation-dependent factors are: (1) form of a preparation (i.e., ointment, cream, lotion); (2) the physical characteristics of a preparation (e.g., its viscosity); and (3) type of container.

31.1.1 The Form of Preparation

When fixed amounts of ointment, cream, and solution are distributed on the skin of volunteers, the ointment was most evenly distributed [2]. Authors believed that this was due to higher viscosity of the ointment. Nevertheless, an additional explanation might be that the volunteers could have much better control (tactile and visual) over the ointment distribution, compared to creams and lotions. The application of the topical preparations, which either evaporate or is absorbed by the skin (lotions, creams), could not be as well controlled as an ointment, which usually stays on the site of application for at least several minutes.

Probably, if creams or lotions were more visible on the skin (e.g., due to added pigment, which could fade in several minutes postapplication), creams and lotions might be applied more evenly, as better control over their distribution could be achieved.

31.1.2 The Physical Characteristics of Preparation

If the amount of the applied topical agents was not fixed, the applied amounts strongly depended on the preparation's viscosity. For instance, a "chemical" type of sunscreen, which was easier to spread on the skin, was applied in amounts up to 50% higher than the "physical" sunscreens of higher viscosity (1.48 mg/cm^2 versus 0.94 mg/cm^2, respectively [3]). Meanwhile, the same study did not reveal statistically significant differences between the amounts of the applied chemical sunscreens with various sun-protective factors (SPF): 8, 15, and 25.

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Note that an earlier study [4] did not demonstrate a statistically significant difference between the amounts of applied cream, ointment, and lotion, in spite of their evidently different viscosity. Probably, this may be explained by more sensitive methods of investigations used in the later study.

31.1.3 Type of Container

A study demonstrated that the dispenser type may influence the amount of medication applied by the patients: the cream contained in an open jar was applied by volunteers much more readily than the same cream from a tube (1.7 mg/cm^2 versus 0.71 mg/cm^2, respectively [4]).

31.2 PATIENT-DEPENDENT FACTORS

Patient-dependent, or behavioral, factors influencing dosing might be divided into three groups: (1) socially mediated factors; (2) factors mediated by medical personnel; and (3) factors mediated by patient's condition.

31.2.1 Socially Mediated Factors

The socially mediated factors are mostly known regarding the use of sunscreens (although we might assume that in case of visible skin lesions, e.g., facial eczema, such factors may also play a role).

It is the social factors which may explain the fact that women use sunscreens more often than men and apply sunscreens more frequently on sun-exposed parts of the body [5,6]. Some associations were also found between use of sunscreens and also self-tanning products by undergraduate students and their close relatives (for facial sunscreens) and romantic partners (for self-tanning preparations) [7]. Household members encouragement increased the frequency of sunscreen use by postal workers of Southern California [6]. At the same time, the people who had relatives with a skin cancer history applied the sunscreens more readily than the people without a family history of skin cancer [6].

Nevertheless, another study [8] demonstrated no significant gender difference in the amount of sunscreen cream applied by students from several European countries—these amounts were uniformly low, the median quantity was 0.39 mg/cm^2, while the amount needed to obtain the nominal SPF must be 1.5 mg/cm^2 (according to the requirements of the German standardization authority—Deutsches Institut für Normung) or 2 mg/cm^2 (according to the requirements of American Standard Association).

No gender differences were also found in the study of application of pharmaceutical topical agents [4], probably because the use of medications was perceived as necessary by both male and female patients, while the use of sunscreens might be perceived more important by females as having a cosmetic effect.

31.2.2 Factors Mediated by Medical Personnel

There is an opinion that little attention is paid to the accurate dosage of topical preparations in terms of explicit instructions to the patient on the quantity of the agent to be applied per unit of time [9]. Other investigators demonstrated that the assistance of medical personnel may change the amount of preparations applied by the patients.

The amount of topical medication (cream) applied by nurses was significantly less (0.91 mg/cm^2) than the quantity of cream applied by patients themselves (1.71 mg/cm^2) [4]. The same authors demonstrated no significant differences between the amounts of topical preparations applied by uninstructed and instructed patients.

Operator-assisted (e.g., nurse) total-body application of cream/ointment resulted in less cream applied, but the cream was spread more equally than in the case of self-application [10]. It was interesting to compare the distribution of the cream/ointment applied by patients themselves at different anatomic sites and the surface areas of the sites (see Table 31.1). Significant differences existed between cream distribution over the region and the real surface of the region, for example, genitoanal area received more than sevenfold the amount of cream in comparison with its surface share.

The application of sunscreens and barrier creams, on the contrary, did reveal the influence of instructions provided by medical personnel.

Though sunscreens are supposed to be used with no instructions or assistance, Azurdia et al. [11] showed that self-applied sunscreens are spread in low mean amounts with great variability: from 0 to 1.2 mg/cm^2 at different anatomic sites (mean amount 0.5 mg/cm^2). Maximum thickness was found on the forehead, cheeks, nose, and chin (1.0 mg/cm^2 or greater), while the mean cream thickness on the temple, ears, lateral, and posterior neck approached zero.

Loesch and Kaplan [12] also showed that periorbital areas, perioral regions, and the ears are rarely covered by sunscreen properly; they suggested some rules for patients to enhance complete sun protection. Subsequently, Azurdia et al. [13] demonstrated that after special instructions the same patients applied 5- to 10-fold higher amounts of sunscreen than prior to instructions.

TABLE 31.1
Distribution of Cream/Ointment by Body Areas

Part of the Body	Cream Amount, %	Surface of Body Area of Adults, % of Total Body Surface
Head	7.6 ± 2.3	9
Arms	22.3 ± 1.6	19
Legs	43.6 ± 2.3	40
Trunk, anterior	14.1 ± 2.0	13
Trunk, posterior	12.4 ± 1.8	18
Genitoanal area	7.4 ± 1.0	1

Source: Schlagel C.A., and Sanborn E.C., *J. Invest. Dermatol.*, *42*, 253–256, 1964.

There was a thorough investigation of distribution of self-applied industrial protective cream in three groups of workers—metal workers, hospital cleaners, and construction workers [14]. Many areas of the hands and forearms were not covered properly by protective cream, which might result in a higher incidence of irritant dermatitis. The authors suggested a special educational program for workers to ensure the proper use of protective cream at the workplace.

Subsequent investigations showed that the uptake of barrier creams was significantly higher among German bakers, who received special training on skin protection, compared to those who did not receive it [15].

31.2.3 Factors Dependent on Patient's Condition

Severe cases of skin diseases may require larger amounts of topical medications, and this is well understood by the patients, so that the patients with more severe cases are ready to apply much larger amounts of topical agents.

It was also demonstrated that, for example, the postal workers with higher skin sensitivity to sun used sunscreens significantly more frequently than their colleagues with lower skin sensitivity [6].

31.3 CONCLUSION

Numerous factors may influence the applied amounts of topical preparations. It is important that the mentioned factors be considered if any conclusion is to be made on effectiveness of a topical preparation—either for a specific patient, or for a group of the patients.

The investigations failed to demonstrate the influence of medical instructions on self-application of topical medications [4]; but in case of preventive agents (barrier creams and sunscreens) such instructions were beneficial [13]. Further studies may provide additional information on the reasons for such a difference.

When clinical trials of topical agents are conducted, and no direct measurement of the applied amounts of the substances is provided, there should always be a proper control of the possible factors influencing the application of the substances on the skin. Otherwise, the obtained data may not be comparable.

Generally, we believe that there remains much room for innovations, which would be able to provide more precise dosing of topical preparations.

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32 Barrier Creams

Hongbo Zhai and Howard I. Maibach

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32.1 INTRODUCTION

Each day the skin is exposed to an infinite number of substances; some may be potentially irritants (e.g., surfactants, cutting oils, acids, and alkalis) or allergens (e.g., poison oak/ivy). Skin barrier function may be damaged due to contact with these materials. Consequentially, irritant contact dermatitis (ICD) and allergic contact dermatitis (ACD) may develop. Minimizing exposure is recommended but often not practicable. In many occupations, such as farmers, forest firefighters, outdoor activities, hospitals, and even households, such encounters are ubiquitous. Therefore, to prevent or reduce the risk of developing ICD and ACD, prophylactic measures are indicated. Application of barrier creams (BC) before or during work may play an important role in the prevention of occupational contact dermatitis and nature hand care as well.

Their efficacy in reducing the developing ICD and ACD has been documented *in vitro* and *in vivo* experimental studies (Frosch et al., 1993a; Lachapelle, 1996; Zhai and Maibach, 1996a, 1999, 2001, 2002; Wigger-Alberti and Elsner, 1998, 2000a,b; Maibach and Zhai, 2000). Yet inappropriate BC application may induce a deleterious rather than a beneficial effect (Goh, 1991a,b; Frosch et al., 1993a–d; Treffel et al., 1994; Zhai and Maibach, 1996b; Lachapelle, 1996). This updated chapter from Zhai and Maibach (2004) emphasizes on BC's terms, mechanism, and other relative topics in this field.

32.2 DEFINITION AND TERMS

BC are designed to prevent or reduce the penetration and absorption of various hazardous materials into skin, preventing skin lesions and other toxic effects from dermal exposure (Orchard, 1984; Frosch et al., 1993a; Lachapelle, 1996; Zhai and Maibach, 1996a,b). BC are also called "skin protective creams" (SPCs) or "protective creams" (PCs), as well as "protective ointments," "invisible glove," "barrier," "protective" or "prework" creams or gels (lotions), "antisolvent" gels, and so on (Guillemin et al., 1974; Mahmoud and Lachapelle, 1985; Loden, 1986; Goh, 1991b; Frosch et al., 1993a). Kresken and Klotz (2003) believe that the term "invisible glove" is incorrect and it might mislead the user. Frosch et al. (1993a) consider SPC a more appropriate terminology since most creams do not provide a real barrier, at least not comparable to stratum corneum. We utilize BC here because this term is in general usage in industry. BC may share characteristics with moisturizers. BC's target is the prevention of external noxious substances penetrating skin, and moisturizers are frequently used for "dry" skin conditions, as well as to maintain healthy skin (Zhai and Maibach, 1998). BC and moisturizers may overlap in chemistry and function.

32.3 REASONS TO UTILIZE BC

Occupational contact dermatitis is the most common work-related injury involving millions of workers worldwide. Avoidance of these irritants or allergens may not be practical for persons whose occupation or activities mandate their working in certain environments. Certain gloves provide protective effects for corrosive agents (acids, alkalis, etc.) (Boman et al., 1982; McClain and Storrs, 1992; Mellstrom et al., 1996; Wigger-Alberti and Elsner, 1998). Protective clothing, as well as other personal devices, also plays a critical role as an important measure in industries (Mathias, 1990; Davidson, 1994). But, protective clothing may trap moisture and occlude potentially damaging substances next to the skin for prolonged periods and increase the likelihood that dermatitis will develop (Mathias, 1990; Davidson, 1994). In practice, BC are recommended only for low-grade irritants (water, detergents, organic solvents, cutting oils) (Frosch et al., 1993a; Zhai and Maibach, 1996b; Wigger-Alberti and Elsner, 1998). The first line of defense against hand eczema

is to wear gloves, but in many professions this is impossible because of the loss of dexterity. In some instances, an alternative would be to utilize BC. BC are also used to protect the face and neck against chemical and resinous dust and vapors (Birmingham, 1969). Many prefer to use BC rather than gloves because they do not want the hand continuously sealed inside a glove that can inhibit skin barrier function (Wigger-Alberti and Elsner, 1998). In addition, many gloves do not resist the penetration of low molecular weight chemicals. Some allergens are soluble in rubber gloves and may penetrate the glove and produce severe dermatitis (Mathias, 1990; Estlander et al., 1996; Wigger-Alberti and Elsner, 1998). Allergy to rubber latex has become a growing problem (Estlander et al., 1996; Wigger-Alberti and Elsner, 1998). Furthermore, due to continuous wearing of gloves, workers can develop serious symptoms (i.e., contact urticaria syndrome) including generalized urticaria, conjunctivitis, rhinitis, and asthma (Amin and Maibach, 1997; Wigger-Alberti and Elsner, 1998).

In addition, BC can be used against the chemical warfare (Chilcott et al., 2005). This chapter focuses on the general occupational industries only.

32.4 MECHANISM OF ACTION AND DURATION

Minimal information exists on the mechanisms of BC's action. The frequently quoted general rule is that water in oil (W/O) emulsions are effective against aqueous solutions of irritants, and oil in water (O/W) emulsions are effective against lipophilic materials (Mathias, 1990; Frosch et al., 1993a; Davidson, 1994; Lachapelle, 1996); exceptions have demonstrated (Frosch et al., 1993c; Frosch and Kurte, 1994). BC may contain active ingredients presumed to work by trapping or transforming allergens or irritants (Frosch and Kurte, 1994; Lachapelle, 1996). Most believe they interfere with absorption and penetration of the allergen or irritants by physical blocking—forming a thin film that protects the skin (Orchard, 1984; Frosch and Kurte, 1994; Marks et al., 1995; Lachapelle, 1996).

To avoid frequent interruptions for reapplication, BC are expected to remain effective for 3 or 4 h. Most manufacturers claim that their products last ~ 4 h. Others suggest use "as often as necessary" (Davidson, 1994). Studies document duration of action—with varying results (Reiner et al., 1982; Boman et al., 1982; Zhai and Maibach, 1996b; Zhai et al., 1999).

32.5 APPLICATION METHODS AND EFFICACY

BC effectiveness may be influenced by application methods (Packham et al., 1994; Wigger-Alberti et al., 1997a,b). A study had been conducted to determine which areas of the hands were likely to be skipped on self-application of BC by a fluorescence technique at the workplace (Wigger-Alberti et al., 1997a); application of BC was incomplete, especially on the dorsal aspects of the hands. Most manufacturers suggest rubbing thoroughly onto skin; to pay special attention to cuticles and skin under nails; to let it dry approximately 5 min; to apply a thin layer of BC to all appropriate skin surfaces three to four times daily. We believe these suggestions are important for BC efficacy.

In vivo and *in vitro* methods have been developed to evaluate the efficacy of BC. Frosch et al. (1993a), Lachapelle (1996), Wigger-Alberti and Elsner (1998, 2000a,b), and Zhai and Maibach (1996a, 1999, 2000, 2001, 2004) have extensively reviewed their efficacy.

32.6 U.S. FOOD AND DRUG ADMINISTRATION MONOGRAPH "SKIN PROTECTANTS"

U.S. Food and Drug Administration (FDA) identified 13 skin protectants for over-the-counter (OTC) products and regulated in Federal Register (1983). These ingredients and concentrations are listed in Table 32.1. The lack of new ingredients suggests research stagnation.

In addition, an OTC lotion (containing quaternium-18 bentonite) against poison ivy, oak, or sumac has been approved as a new drug application by U.S. FDA.

32.7 CONCLUSION

The efficacy of BC in preventing or reducing ICD and ACD has been well documented in many experimental environments. A recent study has shown an excellent result that BC containing a zinc gel significantly reduced skin irritation in volunteers who exhibited type IV hypersensitivity when exposed to latex gloves (Modak et al., 2005). Obviously, BC may inhibit low-grade irritants, but should be not used as a primary protection against high-risk substances as well as corrosive agents. However, inappropriate BC application may exacerbate irritation rather than provide benefit. In particular, using BC

TABLE 32.1
U.S. Food and Drug Administration (FDA) Identified 13 Skin Protectants and Their Concentrations

Ingredients	Concentrations (%)
Allantoin	0.5–2
Aluminum hydroxide gel	0.15–5
Calamine	1–25
Cocoa butter	50–100
Dimethicone	1–30
Glycerin	20–45
Kaolin	4–20
Petrolatum	30–100
Shark liver oil	3
White petrolatum	30–100
Zinc acetate	0.1–2
Zinc carbonate	0.2–2
Zinc oxide	1–25

Note: Most of these entities have been available for decades and suggest research stagnation.

on diseased skin may lead to increased skin irritation (Mathias, 1990; Lachapelle, 1996). People utilizing water, soaps, and detergents daily may benefit by applying BC frequently. Furthermore, BC may also shield skin from chemicals, oils, and other substances, and to make them easier to clean at the end of the workday (Davidson, 1994). To achieve optimal protective effects, BC should be used with careful consideration of the types of substances they are designed to protect against based on specific exposure conditions; also, the proper use of BC should be instructed (Wigger-Alberti et al., 1997a,b).

The ideal BC should be nontoxic, noncomedogenic, nonirritating, nongreasy foam, and colorless. They should keep high efficacy, but not interfere with user's manual dexterity or sensitivity. They should be easy to apply and remove, cosmetically acceptable, and economical. They may be combined with cosmetic benefits, and contain a high proportion of fatty materials (lipids) and can, therefore, also be used for skin care, especially for rough, dry, or chapped skin. Furthermore, the mechanisms of BC's action should be further investigated when evaluating their efficacy. Recent investigative information will hopefully lead to controlled field trials (Berndt et al., 2000; Schnetz et al., 2000), so that we will have clearer insights into when and how to efficiently utilize them.

Taken together, the field now contains an almost critical mass of workers, ideas, and motivation. Considering the magnitude of irritant dermatitis in industry much remains to be done!

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33 OECD Guidelines for Testing of Chemicals

Klaus-Peter Wilhelm and Howard I. Maibach

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33.1 INTRODUCTION

33.1.1 General

1. This publication contains an excerpt of the official OECD guidelines for testing chemicals as adopted by the OECD council.
2. The test guidelines have been developed initially under the OECD chemicals testing program (see points 10–16), and subsequently, since 1981, as provided by the council under the OECD updating program for test guidelines.
3. Whenever testing of chemicals is contemplated, the OECD test guidelines should be consulted. Since the test guidelines have been endorsed by the OECD member countries, their use in the generation of data provides a common basis for the acceptance of data internationally, together with the opportunity to reduce direct and indirect costs to governments and industry associated with testing and assessment of chemicals.
4. Other methods and guidelines not included in this publication may be judged to be appropriate in testing chemicals in certain scientific, legal, and administrative contexts.
5. The OECD council decision on mutual acceptance of data (May 12, 1981; C(81)30) affirms that data generated in one country in accordance with the OECD test guidelines—and additionally in accordance with the OECD principles of good laboratory practice—should be accepted in OECD countries for purposes of assessment and other uses relating to protection of man and the environment. The full text of this decision and the OECD principles of good laboratory practice may be found in the appendix to the OECD guidelines for testing of chemicals.
6. The OECD test guidelines contain generally formulated procedures for the laboratory testing of a property or effect deemed important for the evaluation of health and environment hazards of a chemical. The guidelines vary somewhat in respect of detail, but include all the essential elements, which, assuming good laboratory practice, should enable an operator to carry out the required test.
7. OECD test guidelines are not designed to serve as rigid test protocols. They are instead designed to allow flexibility for expert judgement and adjustments to new developments.
8. It is intended that the OECD test guidelines be used by experienced laboratory staff familiar with the type(s) of testing involved. Proper conduct of testing and associated interpretation of results can only be achieved by appropriately trained personnel with access to equipped laboratory facilities.
9. The loose-leaf system chosen for the guidelines allows for additions and changes to be made when necessary. Information will be circulated when such changes occur resulting from work under the updating program.

33.2 OECD CHEMICALS TESTING PROGRAM

10. The OECD chemicals testing program was launched by the chemicals group in November, 1977. It composed of six expert groups under the leadership of individual member countries. One of these groups, the step system group, worked on phased approaches

to testing and assessment of chemicals (see points 30–32).

11. Five of the groups reviewed the state-of-the-art of methods and produced draft test guidelines. The following areas were covered:
    i. Physicochemical properties (lead country—Germany)
    ii. Effects on biotic systems other than man (lead country—the Netherlands)
    iii. Degradation/accumulation (lead countries—Japan/Germany)
    iv. Long-term health effects (lead country—the United States)
    v. Short-term health effects (lead country—the United Kingdom)
12. Some 300 experts, drawn from academia, government, industry, international organizations, and other sectors, took part in the program. In all, some 50 meetings were held during 1978–1979 under the auspices of the OECD chemicals testing program.
13. To improve the international validation of tests, several methods were subjected to laboratory intercomparison exercises in the chemicals testing program. This work is being continued under the OECD updating program.
14. In December 1979, the five expert groups working on test methods submitted their reports to the OECD. The two groups on health effects submitted a combined report. The reports contained draft test guidelines and an analysis of approaches to testing within the respective areas.
15. During 1980, the draft test guidelines were subjected to an extensive commentary and review process. Member countries were invited to submit comments to the OECD, which were subsequently taken into account by a review panel, established to finalize the product for adoption and printing. The panel worked in close collaboration with the chairman of the expert groups.
16. The review process was concluded by the chemicals group and the environment committee of the organization that endorsed these test guidelines prior to their formal submission to the OECD council.
17. The subject areas covered by the expert groups under the chemicals testing program have largely been kept separate in this publication. Thus, the OECD test guidelines are presented under four different sections:
    i. Physicochemical properties
    ii. Effects on biotic systems other than man
    iii. Degradation/accumulation
    iv. Health effects
18. Each section is preceded by a summary of considerations raised in the individual expert group reports. These summaries reflect some of the major observations and explanations made at the scientific level during the preparatory process. Further, major portions of the expert group reports have been absorbed into the on-going activities of OECD on chemicals.

33.3 OECD UPDATING PROGRAM FOR TEST GUIDELINES

19. In 1981, the OECD updating program for test guidelines was established by member countries in consultation with the commission of the European communities. The aim was to ensure that OECD test guidelines will not become outdated as a result of major changes in the state-of-the-art or scientific advances.
20. The updating program is considering:
    i. Proposals for new or modified tests, which offer conspicuous advantages over those already adopted
    ii. New guidelines, which are being developed in areas not yet covered
    iii. Incorporation of results from the chemicals testing program into OECD test guidelines
    iv. Those matters that need further investigation and research

33.4 OECD PRINCIPLES FOR TESTING AND ASSESSMENT OF CHEMICALS

21. The OECD test guidelines are but one component in an OECD strategy to make testing of chemicals more systematic, relevant, and cost effective within an international framework, which could lead to increased exchange and acceptance of test data between countries. This strategy has been developed with vigor in the organisation during the 1970s, leaving several important questions yet to be resolved.
22. While the OECD test guidelines can properly be used in establishing one effect or property, the guidelines were developed under programs directed toward an integrated and comprehensive approach to testing and assessment. Thus, the OECD council, in 1974 and 1977, developed recommendations, which deal respectively with "The assessment of the potential environmental effects of chemicals" (C[74]215) and "Guidelines in respect to procedures and requirements for anticipating the effects of chemicals on man and in the environment" (C[77]97[Final]).
23. In 1974, the OECD council recommended that prior to marketing of chemicals their potential effects on man and his environment should be assessed.
24. This concern, that assessments should encompass both man and his environment, was reflected in the subject areas chosen for the OECD chemicals testing program, and is also reflected in the disposition of the test guidelines into sections.

25. Some outstanding features with respect to testing and assessment, which derive from the 1977 OECD council recommendations, can be summarized as follows:
    i. Chemical substances—with special emphasis on new substances—should be subjected to systematic assessment for potential effects, in relation to both human and environmental hazard.
    ii. It is possible to determine no more than the likelihood of adverse affects from chemicals, and this can only be done through the application of expert judgement based on methods that are technically practicable, as well as economically acceptable.
    iii. Responsibility for generating and assessing the data necessary to determine the potential effects of chemicals must be part of the overall function and liability of industry.
    iv. A phased approach should be applied in data gathering and assessments.
26. These four principles also provided guidance to the expert groups in their work in the chemicals testing program.
27. The need for expert judgement in testing and assessment has been emphasized throughout the work on chemicals in OECD. The expert groups under the OECD chemicals testing program reaffirmed this need when they selected methods that were regarded as technically practicable and economically acceptable for inclusion into OECD test guidelines.
28. The question of a phased approach to testing and assessment is an important concept, which is under continuing active consideration in OECD within the chemicals testing program. All the expert groups have contributed to the framework of an overall scheme for testing and assessment of chemicals.
29. In their work, the five expert groups on test methods identified steps in which testing and assessment might proceed. The early steps were usually simple in character with the objective of establishing a first indication of hazard. Further steps brought the testing and assessment into a sophisticated and time-consuming range of tests, characterized by increased confidence in the assessments.
30. The step systems group, the sixth expert group established under the chemicals testing program, draws upon the work of the other expert groups and is currently developing an integrated stepwise approach to testing and assessment of chemical hazard to man and his environment.
31. An important outcome of the work of the step systems group is the OECD minimum premarketing set of data (MPD).
32. MPD lists some 35 individual data components that normally would be sufficient to perform a meaningful first assessment of the potential hazard of a chemical.
33. Finally, it should be recognized that elaboration of principles for testing and assessment of chemicals is a continuing process within OECD. This process has been, and remains, possible only through the generous provision of time, knowledge, and enthusiasm from the participating experts, and the active support of member countries.

33.5 ADOPTED TEST GUIDELINES

The complete list of OECD guidelines is available on the OECD webpage (www.oecd.org). Following is a list of those guidelines pertaining to dermal and ocular toxicity.

402 Acute Dermal Toxicity (updated guideline, adopted on February 24, 1987)

404 Acute Dermal Irritation/Corrosion (updated guideline, adopted on April 24, 2002)

405 Acute Eye Irritation/Corrosion (updated guideline, adopted on April 24, 2002)

406 Skin Sensitisation (updated guideline, adopted on July 17, 1992)

410 Repeated Dose Dermal Toxicity: 21/28 day study (original guideline, adopted on May 12, 1981)

411 Subchronic Dermal Toxicity: 90 day Study (original guideline, adopted on May 12th, 1981)

427 Skin Absorption: *In Vivo* Method (original guideline, adopted on April 13, 2004)

428 Skin Absorption: *In Vitro* Method (original guideline, adopted on April 13, 2004)

429 Skin Sensitisation: Local Lymph Node Assay (updated guideline, adopted on April 24, 2002)

430 *In Vitro* Skin Corrosion: Transcutaneous Electrical Resistance Test (TER) (original guideline, adopted on April 13, 2004)

431 *In Vitro* Skin Corrosion: Human Skin Model Test (original guideline, adopted on April 13, 2004)

432 *In Vitro* 3T3 NRU Phototoxicity Test (original guideline, adopted on April 13, 2004)

451 Carcinogenicity Studies (original guideline, adopted on May 12, 1981)

33.6 DRAFT TEST GUIDELINES FOR WHICH COMMENTING PERIOD HAS EXPIRED AND WHICH ARE BEING REVISED OR FINALIZED

434 Acute Dermal Toxicity-Fixed Dose Procedure Draft New Guideline (May 2004)

(Deadline for public comments passed on July 16, 2004)

435 *In Vitro* Membrane Barrier Test Method for Skin Corrosion Draft New Guideline (May 2004)

(Deadline for public comments passed on July 16, 2004)

34 Methods for *In Vitro* Percutaneous Absorption

Robert L. Bronaugh

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34.1 INTRODUCTION

In vitro percutaneous absorption methods are used for various reasons. Often it may be the only ethical way of obtaining human skin absorption data with potentially toxic chemicals. These studies also facilitate simultaneous measurement of skin metabolism, which can be examined without metabolic interference from systemic organs (see Chapter 42). The use of animals is also minimized with *in vitro* studies since many diffusion cells can be assembled from the skin of one animal.

It is important to conduct a study in a way that most closely simulates normal exposure to the chemical of interest. The length of exposure of a chemical in contact with the skin is often assumed to be 24 h unless it is washed off more quickly such as with a shampoo or hair color. Since the vehicle can play a major role in determining the absorption rate, the vehicle used in the absorption study should be similar to that found in normal exposure conditions.

34.2 PRELIMINARY STEPS

It is useful in the planning of studies to have knowledge of the test chemicals solubility and partitioning properties. The log of the octanol/water partition coefficient (Log P) has been used for years as an indicator of percutaneous absorption properties. It is an indicator of the lipophilicity of a chemical, which is a property necessary for it to permeate through the lipid-enriched stratum corneum layer. Water solubility is necessary for permeation through the more aqueous-viable epidermal and dermal tissue. Diminished skin absorption may start to be observed with chemicals of molecular weight above 500 Da (Bos and Meinardi, 2000).

34.3 DIFFUSION CELLS

There are two basic designs of one-chambered diffusion cells—the flow-through cell (Bronaugh and Stewart, 1985) and static cell (Franz, 1975). The one-chambered cell has a chamber (receptor) beneath the skin but is open to the environment above the skin to simulate many exposure conditions. Static diffusion cell systems are simpler in design and frequently based on the Franz diffusion cell. Receptor fluid beneath the skin is manually sampled by removing aliquots periodically for analysis. Besides the cost advantage, another important feature is their availability in a wide range of larger openings for skin that might be needed, for example, in studies with transdermal devices.

A flow-through diffusion cell has an advantage in receptor fluid sampling, which can be done automatically using a fraction collector. The maintenance of cell viability for metabolism studies is facilitated by the continual replacement of receptor fluid. Use of a flow-through cell helps prevent high concentrations of test compound in the receptor fluid that can reduce absorption, and the cell may facilitate partitioning of water-insoluble chemicals from skin.

Special attention may be necessary in measuring the permeability of highly volatile compounds when the skin is not occluded to prevent evaporation. The short walls on the top of some diffusion cells can protect the skin surface from air currents, and it has been suggested that this protection may be responsible for some differences observed between *in vivo* and *in vitro* results (Bronaugh and Maibach, 1985; Bronaugh et al., 1985). Diffusion cells have been designed to collect evaporating material above the surface of the skin (Spencer et al., 1979; Reifenrath and Robinson, 1982). These cells have

proven useful particularly in studies of the effectiveness of mosquito repellents and in studies of volatile compounds that require mass balance determinations.

A two-chambered cell has two chambers of equal volume (often from 2 to 10 mL) that are separated by the skin membrane. Variations of the two-chambered cell have been used for years to create conditions in which the diffusion of a compound in solution can be measured from one side of the membrane to the other (Scheuplein, 1965). An infinite dose (one that is large enough to maintain constant concentration during the course of an experiment) is added to one side of the membrane, and its rate of diffusion across a concentration gradient into a solution on the opposite side is determined. Usually, the solutions on both sides of the membrane are stirred to ensure uniform concentrations. Studies comparing permeation through skin to Fickian diffusion through a membrane are performed in this fashion. The two-chambered cell is useful for studying mechanisms of diffusion through skin. It is also applicable to the measurement of absorption from drug delivery devices where compounds are applied to skin at an infinite dose and a steady-state rate of delivery is desired.

34.4 PREPARATION OF SKIN

Skin that is harvested from human or animal sources may need to be washed prior to further preparation for the diffusion cells. Washing should be carefully conducted with a soap and water solution followed by a water rinse.

Full-thickness skin should generally not be used for absorption studies. All or most of the dermis should be removed to simulate the *in vivo* diffusional barrier layer. Chemicals that are systemically absorbed are taken up into the blood vessels of the papillary dermis directly beneath the epidermis. A dermatome is commonly used to prepare a split-thickness preparation of skin because it can be used for all types of skin and the viability of skin can be maintained (Bronaugh and Collier, 1991). Full-thickness skin (stratum corneum side up) is fixed to a styrofoam block with hypodermic needles. The dermatome is pushed across the skin surface to prepare a layer of skin with much of the dermis removed. A dermatome section of 200–300 μm is satisfactory since thinner preparations are difficult to make without damage to the skin.

Heat treatment is the only other practical method to remove the dermal tissue, but it must be used on nonhairy skin to avoid damage to the barrier during the separation process. Full-thickness skin is submerged in 60°C water for approximately 1 min, and the epidermal and dermal layers can be pulled apart with forceps (Scheuplein, 1965; Bronaugh et al., 1981). All but the most stable enzymes are destroyed by this process.

The use of full-thickness skin is really only then justifiable when using animal skin that is already very thin, such as that from the mouse (400 μm) (Behl et al., 1984) or rabbit. With the skin of other animals, such as the rat (800–870 μm) (Yang et al., 1986), guinea pig, monkey, and pig, full-thickness skin is almost 1 mm in thickness; and with human skin it can be several millimeter thick (Loden, 1985). Therefore, some means should be used to prepare a membrane that more accurately reflects the barrier layer in thickness. This is particularly important when examining a hydrophobic compound, which diffuses slowly through the aqueous viable tissue.

After the skin is assembled in the diffusion cells, barrier integrity should be verified by measuring the absorption of a standard compound, such as tritiated water. A number of laboratories have reported permeability constant (*K*p) values for tritiated water in normal skin (Bronaugh et al., 1986; Dugard et al., 1984), but this procedure requires dosing the skin for 4–5 h for determination of the steady-state absorption of tritiated water. Bronaugh and co-workers developed a 20-min test for tritiated water absorption to give more rapid results without hydration of skin samples (Bronaugh et al., 1986).

34.5 RECEPTOR FLUID

The selection of the receptor fluid has become an increasingly important decision as investigators strive to create *in vitro* conditions that can adequately duplicate the *in vivo* situation. For measuring the absorption of water-soluble compounds, the use of normal saline or an isotonic buffer solution may be sufficient. However, some chemicals are metabolized significantly during the percutaneous absorption process (Yourick and Bronaugh, 2000). The viability of skin can be maintained for 24 h in a flow-through diffusion cell using a physiological buffer as the receptor fluid (Collier et al., 1989). Metabolism and percutaneous absorption can be measured simultaneously as discussed in Chapter 42. The combined information gives a more complete picture of absorption since the actual permeating species are identified.

Sometimes surfactants (Bronaugh and Stewart, 1984) or organic solvents (Scott and Ramsey, 1987) have been added to the receptor fluid in nonviable skin studies to increase the solubility of lipophilic compounds, and thereby promote free partitioning of chemicals from skin into the receptor fluid. Care must be taken to insure that damage to the skin barrier does not occur. The effectiveness of these methods will likely vary with the solubility properties of the test compound.

More recently, studies have been conducted to examine the significance of test material left in the skin at the end of an absorption study (see Section 34.7).

34.6 TERMINATION OF EXPERIMENT

At the end of a study, the unabsorbed material is washed from skin surface usually using a soap and water solution. Organic solvents have sometimes been used for more lipophilic material. For cosmetic products, removal from the skin is normally accomplished with a soap and water wash.

Barrier integrity may be rechecked with tritiated water or other standard chemical if damage to the barrier is suspected.

The washing procedure itself may be damaging to the skin unless it is carefully accomplished.

34.7 DETERMINATION OF ABSORPTION

The determination of systemic percutaneous absorption is sometimes controversial in an *in vitro* diffusion cell study. Since the skin membrane can sometimes serve as a reservoir for absorbed material, measurement of the absorbed compound appearing in the receptor fluid alone may not be an accurate determination of systemic skin absorption. Both skin and receptor fluid levels should be measured at the end of a study. If determination of systemic absorption is desired, it is not sufficient to simply measure the receptor fluid levels. If significant amounts remain in skin, additional studies may be necessary to determine if the material in skin will eventually be systemically absorbed (see discussion later). Also, skin levels must be known to determine mass balance at the end of the experiment. Recoveries of at least 90% should be obtained unless the test compound is volatile.

Skin can be fractionated to observe localization in different layers. The stratum corneum layer can be removed from the surface of the skin by successive stripping with 10 or more pieces of cellophane tape (Kraeling and Bronaugh, 1997). Individual variation has been reported in the number of strips necessary presumably due to differences in the pressure applied to the tape and differences in the tape itself. The epidermal and dermal layers can be separated with heat as previously described in the preparation of skin.

The guidelines for skin absorption studies recommended by the European Union's Scientific Committee on Consumer Products (SCCP) require that material remaining in the viable skin levels (exclusive of stratum corneum) be considered as systemically absorbed (SCCP, 2006). An exception to this requirement could be made if documentation is provided that irreversible binding of an ingredient occurs to the viable epidermis and that the ingredient is subsequently eliminated by desquamation. The Organization for Economic and Cultural Development (OECD) guideline 428 for *in vitro* skin absorption studies states that all material remaining in skin (including the stratum corneum) may need to be considered as systemically absorbed unless additional studies show that there is no eventual absorption (OECD, 2004).

For example, the lipophilic fragrance ingredient, musk xylol, was shown to be absorbed through hairless guinea pig and human skin (Hood et al., 1996). However, substantial amounts of the fragrance were found in the skin at the end of the 24-h studies (Table 34.1). An additional study was conducted that showed that significant amounts of the material in the skin at 24 h diffused into the receptor fluid in the next 48 h. These results suggest that 24-h receptor fluid values alone do not adequately estimate systemic absorption of musk xylol.

Other compounds have been found to form extensive skin reservoirs of absorbed material during 24 h studies; however, in some instances, extended absorption studies conducted for an additional 48 h have found little or no additional penetration of test material from skin into the receptor fluid. At the end of a 24-h disperse blue 1 absorption study in human skin, 11.1% of the dose applied in a hair dye vehicle remained in skin, and only 0.2% of the applied dose had penetrated into the receptor fluid (Yourick et al., 2004). Only 0.7% of the applied dose of diethanolamine was absorbed through human skin in 24 h from a lotion vehicle while 11.9% was found remaining in skin at the end of the study (Table 34.2) (Kraeling et al., 2004). For both compounds, extended absorption studies suggested that the test material remaining in skin was not available for systemic absorption.

When a substantial reservoir of test material is found in skin at the end of an absorption study, it is important to determine the potential significance of this material for systemic absorption. Conducting an extended absorption study as mentioned above or performing an *in vivo* absorption study can help determine the systemic fate of material in the skin reservoir at the end of an absorption study.

TABLE 34.1
Percentage of Applied Dose Absorbed of Musk Xylol from Oil-in-Water Emulsion and Methanol Vehicles in 24 h

	Hairless Guinea Pig Skin[a]		Human Skin[b]	
	Oil-in-Water Emulsion	Methanol	Oil-in-Water Emulsion	Methanol
Receptor fluid	32.1 ± 1.3	25.8 ± 1.2	4.1 ± 0.7	1.0 ± 0.2
Skin	22.9 ± 2.7	18.8 ± 2.2	17.3 ± 2.3	21.3 ± 0.8
Total absorbed	55.0 ± 2.1	44.6 ± 2.4	21.5 ± 3.0	22.3 ± 0.8
24-h skin wash	24.9 ± 1.4	5.5 ± 0.3	46.9 ± 3.2	25.9 ± 1.3
Total recovery	80.5 ± 1.9	50.4 ± 2.5	68.5 ± 2.9	45.7 ± 3.3

[a] Values are the mean ± SE of four determinations in each of three animals.
[b] Values are the mean ± SE of four or five determinations from two human subjects.

TABLE 34.2
Diethanolamine Penetration in Human Skin from a Lotion Vehicle

	Percentage of Applied Dose Penetrated		
Time of study (h)	24	48	72
Receptor fluid	0.7 ± 0.1	1.2 ± 0.1	1.7 ± 0.5
Stratum corneum	4.2 ± 0.2	3.3 ± 0.1	3.7 ± 0.3
Epidermis and dermis	7.7 ± 0.9	7.4 ± 1.3	7.4 ± 0.9
Total in skin	11.9 ± 0.7	10.7 ± 1.3	11.1 ± 0.6
Total penetration	12.5 ± 0.5	11.9 ± 1.3	12.8 ± 1.1
Recovery	95.2 ± 3.0	93.3 ± 3.4	96.6 ± 3.0

Values are the mean ± SEM of three replicates in each of three donor pieces of human skin. Unabsorbed diethanolamine washed from the surface of skin at 24 h in all three studies. Corresponding values at the different study times are not significantly different (ANOVA, $p < 0.05$).

34.8 EXPRESSION OF PERCUTANEOUS ABSORPTION

Percutaneous absorption values are frequently expressed in terms of percentage of the applied dose absorbed. But these values are valid only at the concentration of the compound tested. Permeability constants are sometimes determined by dividing the steady-state absorption rate by the applied concentration of test compound. A permeability constant is, therefore, a normalized rate constant and is sometimes used to determine skin absorption at various doses. Errors can be introduced in these calculations if the initial lag-time in absorption is ignored and because absorption does not always increase in a linear fashion. Expression of absorption data in terms of rate of penetration (for example, $\mu g/h/cm^2$ skin) can also be useful information for evaluating skin penetration.

34.9 CONCLUSION

There are many methodological decisions to make in conducting an *in vitro* percutaneous absorption study. Careful attention to details should be given when developing a protocol so that the conditions necessary for the study of absorption (and possibly metabolism) of a particular test compound are included.

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35 Percutaneous Absorption of Hazardous Substances from Soil and Water

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35.1 INTRODUCTION

Contamination of soil and water (ground and surface water) and the transfer of hazardous chemical is a major concern. When the large surface area of skin is exposed to contaminated soil and water (work, play, swim, daily bath), skin absorption may be significant. Brown et al. (1984) suggested that skin absorption of contaminants in water has been underestimated and that ingestion may not constitute the sole, or even the primary route of expense. Soil has become an environmental depository for potentially hazardous chemicals. Exposure through work in pesticide-sprayed areas on chemical dump sites seems obvious. However, there may be hidden dangers in weekend gardening or in the child's play area.

This chapter demonstrates potential risk from contaminated soil and water, and discusses potential error in dependence on model systems without validation.

35.2 PERCUTANEOUS ABSORPTION

35.2.1 Solvents

Numerous sites have significant levels of organic contaminants in soil, which are either slowly released or degraded, providing a potential long-term source for chemical exposures. Remediation clean-up cost varies dramatically with the level to which soil must be decontaminated. However, a difficulty in establishing soil clean-up level stems, in part, from our lack of knowledge of the dermal bioavailability of chemicals following exposure to environmental mediums. Compared to dermal exposures with neat or aqueous compound, little is understood about the dermal bioavailability of solvents in soil, dust, sludge, or sediment matrices. A method has been developed to determine dermal uptake of solvents under nonsteady state conditions using real-time breath analysis in rats, monkeys, and human volunteers. The exhaled breath was analyzed using an ion-trap mass spectrometer, which can continually quantitate chemicals in the exhaled breath stream in the 1–5 ppb range. The resulting exhaled breath data were evaluated using physiologically based pharmacokinetic (PBPK) models to estimate dermal permeability constants (K_p), under various exposure conditions. Exposures have been conducted comparing the impact of exposure matrix (soil versus water), occlusion versus nonocclusion, and species-differences on the percutaneous absorption of methyl chloroform, trichloroethylene (TCE), and pentachloroethylene. Studies have demonstrated that rat skin is roughly 40 times more permeable than human skin, that bioavailability is decreased when exposures are in a soil versus aqueous matrix, and that under nonoccluded exposure conditions, the majority of the compound is lost to volatilization and unavailable for absorption. These results have clearly illustrated that the methodology was sufficiently sensitive to enable the conduct of animal and human dermal studies at

low exposure concentrations over small body surface areas, for short periods of time.

Table 35.1 summarizes PBPK estimates for solvent human *in vivo* dermal absorption. Hand immersion treatment is a volunteer sitting comfortably with his/her hand immersed in a bucket of water or soil containing one of the solvents. The volunteer wears a facemask. The volunteer inhales fresh air from an air tank. The mask has a special device that switches between inhalation and exhalation. Thus the volunteer exhales through a different pathway such that the exhaled breath goes to a tandem ion-trap mass spectrometer (MS/MS) coupled to a computer which records and can display real time (every few second if wanted) the solvent concentration in the exhaled breath (Poet et al., 2000a,b, 2002).

Table 35.2 gives PBPK model estimates for the dermal absorption of TCE in rats. Estimated permeability constants are listed. Generally, solvent dermal absorption is less for humans than for rats. In both species, solvent absorption is less from soil than from water. This may be due to water's ability to retain solvent within a matrix on the skin better than with soil.

The combination of real-time breath analysis and PBPK modeling provides an opportunity to effectively follow the changing kinetics of uptake, distribution, and elimination phases of a compound throughout a dermal exposure. The sensitivity of the ASGDI-MS/MS system for exhaled-breath analysis is pivotal in enabling studies wherein human volunteers are exposed to low levels of compounds for short periods of time. This real-time, *in vivo* method is suitable for studying the percutaneous absorption of volatile chemicals, and allows exposures to be conducted under a variety of exposure conditions, including occluded versus nonoccluded, rat versus monkey versus human, and soil versus water matrices (Thrall et al., 2000).

TABLE 35.1
PBPK Model Estimates for Human *In Vivo* Dermal Absorption

Solvent	Treatment	K_p(cm/h)
Methylchloroform (TCA)	Water hand immersion	0.0063 ± 0.0006
Trichloroethylene (TCE)	Soil hand immersion	0.0015 ± 0.0005
	Water patch	0.019 ± 0.001
	Soil hand immersion	0.0074 ± 0.000
	Soil patch	0.0043 ± 0.002
Perchlorolthylene (PCE)	Soil hand immersion	0.0009 ± 0.0003

TABLE 35.2
PBPK Model Estimates for the Dermal Absorption of TCE in Rats

Exposure Concentration	K_p^a (cm/h)	Amount Absorbed (mg)	Total TCE Recovered[b] (%)
Occluded water (mg/L)			
1600	0.31 ± 0.018	7.5 ± 1.4	100 ± 5.2
600	0.30 ± 0.006	2.7 ± 0.4	103 ± 5.1
Average	0.31 ± 0.014	102 ± 5.6	
Nonoccluded soil (mg/kg)			
40,600	0.087 ± 0.002	1.5 ± 1.4	98 ± 8.8
20,300	0.085 ± 0.003	7.3 ± 2.7	97 ± 5.7
5000	0.085 ± 0.003	1.7 ± 0.8	101 ± 1.4
Average	0.086 ± 0.003	99 ± 6.0	
Occluded soil (mg/kg)			
15,600	0.090 ± 0.003	40 ± 15	99 ± 2.2
5300	0.089 ± 0.002	14 ± 3.7	99 ± 2.4
Average	0.090 ± 0.002	99 ± 1.0	

[a] Water K_p values are significant from soil ($p < .01$) for both occluded and nonoccluded studies. There is no significant difference in K_p between occluded and nonoccluded soil exposures.

[b] The total TCE recovered was calculated from percent absorbed (estimated from PBPK model), percent remaining in media (soil or water), and percent in charcoal path, where appropriate (as measured using GC headspace analysis) ± SD. The amount absorbed by the body for nonoccluded soil exposure is for a 3 h exposure ($n = 3$) ± SD.

35.2.2 Organic Chemicals

35.2.2.1 DDT, Benzo[a]pyrene, Chlordane, Pentachlorophenol, 2,4-D

Table 35.3 gives the *in vitro* (human skin) and *in vivo* (rhesus monkey) percutaneous absorption of organic chemicals from soil and a comparative vehicle (water or solvent, depending on vehicle). The soil is the same source (Yolo County) for all chemicals. For each chemical the concentration of mass (μg) per unit skin area (cm^2) is the same for each vehicle. It should be pointed out that chemical selection was done according to chemical interest, as expressed by Cal EPA and US EPA. Thus, the chemicals exhibit high log P octanol/water partition

TABLE 35.3
***In Vitro* and *In Vivo* Percutaneous Absorption of Organic Chemicals**

Compound	Vehicle	Percent Dose *In Vitro* Skin	Percent Dose *In Vitro* Receptor Fluid	*In Vivo*
DDT	Acetone	18.1 ± 13.4	0.08 ± 0.02	18.9 ± 9.4
	Soil	1.0 ± 0.7	0.04 ± 0.01	3.3 ± 0.5
Ben[a]pyrene	Acetone	23.7 ± 9.7	0.09 ± 0.06	51.0 ± 22.0
	Soil	1.4 ± 0.9	0.01 ± 0.06	13.2 ± 3.4
Chlordane	Acetone	10.8 ± 8.2	0.07 ± 0.06	6.0 ± 2.8
	Soil	0.3 ± 0.3	0.04 ± 0.05	4.2 ± 1.8
Pentachloro phenol	Acetone	3.7 ± 1.7	0.6 ± 0.09	29.2 ± 5.8
	Soil	0.11 ± 0.04	0.01 ± 0.00	24.4 ± 6.4

TABLE 35.4
Octanol/Water Partition Coefficients of Compounds

Compounds	Log *P*
DDT	6.91
Benzo[a]pyrene	5.97
Chlordane	5.58
Pentachlorophenol	5.12
2,4-D	2.81
PCBs	Mixture
Aroclor 1242	(High log P)
Aroclor 1254	(High log P)

TABLE 35.5
***In Vitro* and *In Vivo* Percutaneous Absorption of PCBs**

Compound	Vehicle	Percentage Dose *In Vitro* Skin	*In Vitro* Receptor Fluid	*In Vivo*
PCBs (1242)	Acetone	–	–	21.4 ± 8.5
	TCB	–	–	18.0 ± 8.3
	Mineral oil	6.4 ± 6.3	0.3 ± 0.6	20.8 ± 8.3
	Soil	1.6 ± 1.1	0.04 ± 0.05	14.1 ± 1.0
PCB (1254)	Acetone	–	–	14.6 ± 3.6
	TCB	–	–	20.8 ± 8.3
	Mineral oil	10.0 ± 16.5	0.1 ± 0.07	20.4 ± 8.5
	Soil	2.8 ± 2.8	0.04 ± 0.05	13.8 ± 2.7

coefficients, rather than a range of log Ps (Table 35.4). The *in vivo* human skin percutaneous absorption is expressed as chemical percent dose in receptor fluid accumulation and skin content. Chemicals with higher log Ps are lipophilic and, therefore, are not soluble in biological fluid or receptor fluid (plasma, buffered saline) (Wester et al., 1990a,b, 1992a).

Receptor fluid (human plasma) accumulation of DDT was negligible in the *in vitro* study due to solubility restriction. Human skin content was 18.1% dose from acetone vehicle. *In vivo* absorption in the rhesus monkey was 18.9% dose from acetone vehicle. These values are comparable to the published 10% dose absorbed *in vivo* in man from acetone vehicle. Percutaneous absorption from soil was predicted to be 1.0% dose in human skin *in vitro* and a comparative 3.3% dose *in vivo* in rhesus monkey.

In vivo percutaneous absorption of benzo[a]pyrene is high—51.0% reported here for rhesus monkey and 48.3% (Bronaugh et al., 1986) and 35.3% (Yang et al., 1989) for rat. Benzo[a]pyrene absorption from soil was approximately one-fourth that of solvent vehicle (Wester et al., 1990a).

For chlordane, pentachlorophenol, and 2,4-D, the *in vivo* percutaneous absorption in rhesus monkey from soil was equal to or slightly less than that obtained from solvent vehicle (Table 35.3). Validation to man *in vivo* is available for 2,4-D, where the percutaneous absorption is the same for rhesus monkey and man. *In vitro* percutaneous absorption is variable, probably due to solubility problems relative to high lipophilicity.

35.2.3 PCBs

Table 35.5 gives the *in vitro* and *in vivo* percutaneous absorption of PCBs (Wester et al., 1993a). As with the other organic chemicals with high log P, receptor fluid accumulation *in vitro* was essentially nil. Skin accumulation *in vitro* did exhibit some PCB accumulation. *In vivo* PCB percutaneous absorption for both Aroclor 1242 and 1254 was (1) high, ranging from 14 to 21%, and (2) generally independent of formulation vehicle. Thus, PCBs have a strong affinity for skin and are relatively easily absorbed into and through skin. Figure 35.1 summarizes absorption from solvent and soil.

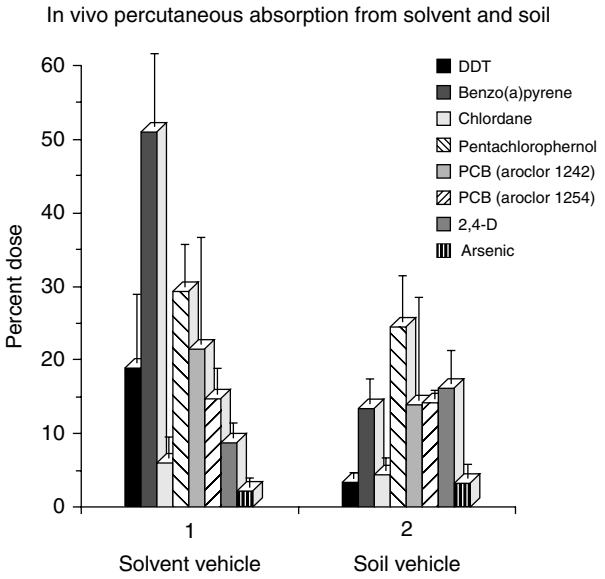


FIGURE 35.1 In percutaneous absorption of several hazardous substances from soil and solvent (either acetone or water), overall, soil reduced absorption to about 60%, compared to solvent. However, the absorption of some compounds is the same for soil and solvent.

35.2.4 Metals

35.2.4.1 Arsenic, Cadmium, Mercury

Selected salts of arsenic, cadmium, and mercury are soluble in water, and thus are amenable to *in vitro* percutaneous absorption with human skin. Arsenic absorption *in vitro* was 2.0% (1.0% skin plus 0.9% receptor fluid), and the same *in vivo* in rhesus monkey. Absorption from soil was equal to (*in vivo*) or approximately one-third (*in vitro*). Cadmium and mercury both accumulate in human skin, and are slowly absorbed into the body. (It should be noted that *in vivo* studies with cadmium and mercury are difficult to perform; cadmium accumulates in the body and mercury is not excreted via urine.) Note the high skin content with cadmium and mercury (Table 35.6) (Wester et al., 1992a, 1993b).

TABLE 35.6
***In Vitro* and *In Vivo* Percutaneous Absorption of Metals**

Compound	Vehicle	Skin	Percent Dose *In Vitro* Receptor Fluid	*In Vivo*
Arsenic	Water	1.0 ± 1.0	0.9 ± 1.1	2.0 ± 1.2
	Soil	0.3 ± 0.2	0.4 ± 0.5	3.2 ± 1.9
Cadmium	Water	6.7 ± 4.8	0.4 ± 0.2	–
	Soil	0.09 ± 0.03	0.03 ± 0.02	–
Mercury	Water	28.5 ± 6.3	0.07 ± 0.01	–
	Soil	7.9 ± 2.2	0.06 ± 0.01	–

TABLE 35.7
***In Vitro* Receptor Fluid versus *In Vivo* Percutaneous Absorption**

Compound	Vehicle	Percent Dose *In vitro* Receptor Fluid	*In vivo*
DDT	Acetone	0.08 ± 0.02	18.9 ± 9.4
	Soil	0.04 ± 0.01	3.3 ± 0.5
Benzo[a]pyrene	Acetone	0.09 ± 0.06	51.0 ± 22.0
	Soil	0.01 ± 0.06	13.2 ± 3.4
Chlordane	Acetone	0.07 ± 0.06	6.0 ± 2.8
	Soil	0.04 ± 0.05	4.2 ± 1.8
Pentachlorophenol	Acetone	0.6 ± 0.09	29.2 ± 5.8
	Soil	0.01 ± 0.00	24.4 ± 6.4
PCBs (1242)	Acetone	–	21.4 ± 8.5
	TCB	–	18.0 ± 8.3
	Mineral oil	0.3 ± 0.6	20.8 ± 8.3
	Soil	0.04 ± 0.05	14.1 ± 1.0
PCBs (1254)	Acetone	–	14.6 ± 3.6
	TCB	–	20.8 ± 8.3
	Mineral oil	0.1 ± 0.07	20.4 ± 8.5
	Soil	0.04 ± 0.05	13.8 ± 2.7
2,4-D	Acetone	–	2.6 ± 2.1
	Soil	0.02 ± 0.01	15.9 ± 4.7
Arsenic	Water	0.9 ± 1.1	2.0 ± 1.2
	Soil	0.03 ± 0.5	3.2 ± 1.9
Cadmium	Water	0.4 ± 0.2	–
	Soil	0.03 ± 0.02	–
Mercury	Water	0.07 ± 0.01	–
	Soil	0.06 ± 0.01	–

35.3 *IN VITRO* DIFFUSION VERSUS *IN VIVO*

Regulatory agencies have developed an affinity for a calculated permeability coefficient (K_p) for risk assessment. Permeability coefficients are easily determined from the time course of chemical diffusion from a vehicle (water, soil) across the skin barrier into a receptor fluid. Table 35.7 compares *in vitro* diffusion receptor fluid absorption with *in vivo* percutaneous absorption. Receptor fluid accumulation for the higher log P chemicals (Table 35.4) is negligible. This is due to basic chemistry—the compounds are not soluble in the water-based receptor fluid. Based on these receptor fluid accumulations, these chemicals are not absorbed by skin. Risk assessment would contain an extreme false-negative component. That point where the diffusion system and receptor fluid accumulation gives a true K_p or manufactures a false K_p has not been determined. Regulatory agents should have some *in vivo* validation before blindly accepting an *in vitro* K_p.

35.4 SOIL LOAD

A popular assumption is that only the fine particles of soil stick to the skin transfer contaminants from the soil to the skin. This is the monolayer theory. If it was only the fine soil particles, then all of the data shown in this chapter could not exist, because the fine particles were not used (sieved out for laboratory personnel safety reasons). Besides, contaminants will transfer between large surfaces (table, couch, etc.) and skin, even between people. And, certainly, the first soil monolayer to contact the skin during planting of the first rose bush will not be the same monolayer after planting the 20th bush. However, the computer model needs the monolayer, therefore, it has to exist. Table 35.8 shows the effect of soil load. Note the chemical concentration was kept constant while soil load was varied.

35.5 DISCUSSION

The evolution of skin resulted in a tissue that protects precious body fluids and constituents from excessive uptake of water and contaminants in the external environment. The outermost surface of the skin that emerged for human is the stratum corneum, which restricts but does not prevent penetration of water and other molecules. This is a complex lipid–protein structure that is exposed to contaminants during bathing, swimming, and exposure to the environment. Industrial growth has resulted in the production of organic chemical and toxic metals whose disposal resulted in contamination. When an adult settles into a tub or a child sits

TABLE 35.8
Effect of Soil Load on 2,4-D Percutaneous Absorption

System	Soil Load[a] (mg/cm²)	Percent Dose Absorbed[b]
In vivo, rhesus monkey	1	9.8 ± 4.0
	40	15.9 ± 4.7
In vitro, human skin	5	1.8 ± 1.7
	10	1.7 ± 1.3
	40	1.4 ± 1.2

[a] Concentration of 2,4-D chemical per cm² skin area was kept constant, while soil load per cm² skin area was varied.

[b] *In vivo* percutaneous absorption measured by urinary ^{14}C accumulation; *in vitro* absorption determined by ^{14}C skin content.

in the dirt for a day of play, the skin (the largest organ of the body) acts as a lipid sink (stratum corneum) for the lipid-soluble contaminants. Skin also serves as transfer membrane for water and whatever contaminants may be dissolved in it. It is most important to note that (1) water transfers through skin and can carry chemicals, and (2) the outer layer of skin is lipid in nature. Thus, highly lipophilic chemicals such as DDT, PCBs, and chlordane residing in soil will quickly transfer to skin. Percutaneous absorption can be linear, orderly, and predictive (a measured flux from water). However, evidence exists that chemicals may transfer to skin with short-term exposure.

Regulators should be cautious as *in vitro* and computer models are developed for risk assessment. Validation is needed to avoid false negative assessment.

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36 Pesticide Percutaneous Absorption and Decontamination

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36.1 INTRODUCTION

Percutaneous absorption is a primary focal point for dermatotoxicology and dermatopharmacology. Local and systemic toxicity depend on a chemical penetrating the skin. The skin is both a barrier to absorption and a primary route to the systemic circulation. The skin's barrier properties are impressive. Fluids and precious chemicals are reasonably retained within the body; at the same time hundreds of foreign chemicals are restricted from entering the systemic circulation. Even with these impressive barrier properties, the skin is a primary body organ that contacts the environment and is a route by which many chemicals enter the body. Some chemicals applied to the skin have proved to be toxic. These include pesticides, which in actuality are designed poisons.

Table 36.1 summarized the 30-year lesson with parathion. Absorption of parathion was established for human skin contact, but other species similarly absorb the compound. Mathematical models based on quantitative structure activity now can predict a human skin permeability coefficient, but the accuracy of the predicted coefficient is not fully validated to *in vivo* man. Skin absorption amounts combined with toxicity data can predict potential human health hazard. Figure 36.1 shows human systemic parathion from dermal exposure. Parathion is predicted to be lethal for not only total systemic absorption but also for exposure to limited regions. The LD_{50} used for parathion is 14 mg/kg. Given a body weight of 70 kg, the systemic absorption of 980 mg might result in 50% mortality. Thus, parathion lethal toxicity levels can be reached at 8 h and longer exposures. This was unfortunately validated in the agricultural fields of California and elsewhere.

36.2 PERCUTANEOUS ABSORPTION METHODOLOGY

36.2.1 Absolute Topical Bioavailability

The only way to determine the absolute bioavailability of topically applied compound is to measure the compound by specific assay in blood or urine after topical and intravenous administration. This is extremely difficult to do in plasma because concentrations after topical administration are often low. However, as advances in analytical methodology bring forth more sensitive assays, estimates of absolute topical bioavailability will become more available (Wester and Maibach, 1999).

36.2.2 Radioactivity in Excreta

Percutaneous absorption *in vivo* is usually determined by the indirect method of measuring radioactivity in excreta after topical application of the labeled compound. In human studies, plasma levels of compound are extremely low after

Adapted (with permission) from Robert Krieger, *Handbook of Pesticide* Toxicology, Academic Press, 2001.

TABLE 36.1
Summary: Parathion Percutaneous Absorption

Parathion

O,O-diethyl O-(4-nitrophenyl) phosphorothioate
Other names: ethylparathione, parathion-ethyl
CAS: 56-38-2; Mol. Wt. 291.26
Molecular formula: $C_{10}H_{14}NO_5PS$
Nonsystemic contact and stomach-acting insecticide and acaricide with some fumigant action
Nonphytotoxic except to some ornamentals and under certain weather conditions. Absorption takes place readily through any portal. Fatal human poisoning has followed skin exposure

Skin Absorption (Species)

Human (forearm); solvent: acetone; 10%, 5 days (excretion analysis) (Feldmann and Maibach, 1974)
Human: solvent: acetone; forearm 8.6%, palm 11.8%, foot 13.5%, abdomen 18.5%, hand, dorsum 21%, forehead 36.3%, axilla 64%, jaw 33.9%, fossal cubitalis 28.4%, scalp 32.1%, ear cannal 46.6%, scrotum 101.6% (Maibach et al., 1974)
Rat (dermal); solvent: acetone; 59%, 1 h (patch) (Knaak et al., 1984)
Mouse (dermal); no solvent; 1.4%, 1 h (excretion analysis). Mouse (dermal); solvent: acetone; 32%, days (patch) (Marty, 1976)
Frog (dermal); solvent: acetone; 33%, 1 h (patch) (Shah et al., 1983)
Quail (dermal); solvent: acetone; 40%, 1 h (patch) (Shah et al., 1983)
Roach (dermal); solvent: acetone; 14%, 1 h (patch) (Shah et al., 1983)
Hornworm (dermal); solvent: acetone; 8%, 1 h (patch) (Shah et al., 1983)

Skin Absorption (Computer Model)

kp (cm/h)	Log P (K_{ow})
1.59×10^{-2}	3.83

Based on the formula: log kp = –2.74 + [0.71 × log P (K_{ow})] – [0.0061 × MW]; where kp = permeability coefficient, P (K_{ow}) = partition coefficient in octanol compared to water, MW = molecular weight (Guy and Plotts, 1992), and P (K_{ow}) = partition coefficient in octanol compared to water

Toxicity

Rat
Oral male LD50: 13–15 mg/kg [105,109]
Oral female LD50: 3–3.6 mg/kg [105,109]
Skin male LD50: 21 mg/kg [105]
Skin female LD50: 6.8 mg/kg [105]

Source: Krieger, R. in *Handbook of Pesticide Toxicology*, Academic Press, 2001. With permission.

topical application, often below assay detection level; so it is necessary to use trace methodology. The compound, usually labeled with ^{14}C or tritium, is applied and the total amount of radioactivity excreted in urine or urine plus feces is determined. The amount of radioactivity retained in the body or excreted by some route not assayed (CO_2, sweat) is corrected for by determining the amount of radioactivity excreted after parenteral administration. This final amount of radioactivity is then expressed as the percentage of applied dose that was absorbed (Feldmann and Maibach, 1974).

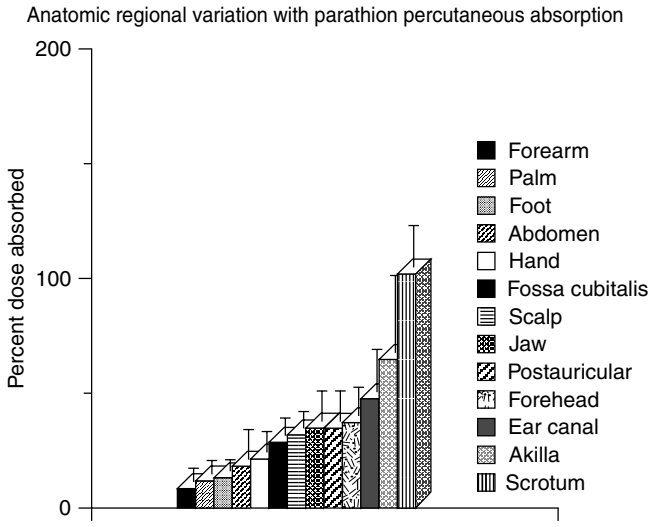


FIGURE 36.1 Simulated parathion human skin exposure to regions of the body. As early as 8 h following exposure lethality is possible. A 24 h lethality is possible if only certain body regions are exposed; example is head and neck to a field worker. (Adapted from Krieger, R. *Handbook of Pesticide Toxicology*, Academic Press, 2001. With permission.)

The equation used to determine percutaneous absorption is:

$$\text{Absorption (\%)} = \frac{\text{total radioactivity after topical administration}}{\text{total radioactivity after parenteral administration}} \times 100$$

36.2.3 Skin Flaps

The methodology is to surgically isolate a portion of skin so that a singular blood supply is created to collect blood containing the chemical that has been absorbed through skin. The skin flap can be used to study percutaneous absorption *in vivo* or *in vitro*. The absorption of chemicals through skin and metabolism within the skin can be determined by assay of the perfusate (Wester and Maibach, 1997).

36.2.4 Stripping Method

The stripping method determines the concentration of chemical in the stratum corneum during an application period and predicts the percutaneous absorption of that chemical. The chemical is applied to skin of animals or humans, and at various skin application times the stratum corneum is removed by successive tape application and removal. The tape strippings are assayed for chemical content. Pharmacokinetic Cmax, Tmax, and area-under-curve (AUC) parameters can be calculated for stratum corneum bioavailability.

36.2.5 Biological Response

Another *in vivo* method of estimating absorption is to use a biological or pharmacological response. Here, a biological assay is substituted for a chemical assay and absorption is estimated. An obvious disadvantage to the use of biological response is

TABLE 36.2
***In Vitro* versus *In Vivo* Percutaneous Absorption**

			Percent Dose		
			In Vitro		
Compound	**Log *P*[a]**	**Vehicle**	**Skin**	**Receptor Fluid**	***In Vivo***
DDT	6.9	Acetone	18.1 ± 13.4	0.08 ± 0.02	18.9 ± 9.4
		Soil	1.0 ± 0.7	0.04 ± 0.01	3.3 ± 0.5
Benzo[a]pyrene	5.97	Acetone	23.7 ± 9.7	0.09 ± 0.06	51.0 ± 22.0
		Soil	1.4 ± 0.9	0.01 ± 0.06	13.2 ± 3.4
Chlordane	5.58	Acetone	10.8 ± 8.2	0.07 ± 0.06	6.0 ± 2.8
		Soil	0.3 ± 0.3	0.04 ± 0.05	4.2 ± 1.8
Pentachlorophenol	5.12	Acetone	3.7 ± 1.7	0.6 ± 0.09	29.2 ± 5.8
		Soil	0.11 ± 0.04	0.01 ± 0.00	24.4 ± 6.4
PCBs (1242)	High	Acetone			21.4 ± 8.5
		TCB			18.0 ± 8.3
		Mineral oil	6.4 ± 6.3	0.3 ± 0.6	20.8 ± 8.3
		Soil	1.6 ± 1.1	0.04 ± 0.05	14.1 ± 1.0
PCBs (1254)	High	Acetone			14.6 ± 3.6
		TCB			28.0 ± 8.3
		Mineral oil	10.0 ± 16.5	0.1 ± 0.07	20.4 ± 8.5
		Soil	2.8 ± 2.8	0.04 ± 0.05	13.8 ± 2.7
2,4 Dichlorophenoxy-acetic acid (2,4-D)	2.81	Acetone			8.6 ± 2.1
		Soil	1.6 ± 0.2	0.02 ± 0.01	15.9 ± 4.7
Arsenic		Water	1.0 ± 1.0	0.9 ± 1.1	2.0 ± 1.2
		Soil	0.3 ± 0.2	0.4 ± 0.5	3.2 ± 1.9
Cadmium		Water	6.7 ± 4.8	0.4 ± 0.2	
		Soil	0.09 ± 0.03	0.03 ± 0.02	
Mercury		Water	28.5 ± 6.3	0.07 ± 0.01	
		Soil	7.9 ± 2.2	0.06 ± 0.01	

[a] Note that a log *P* of 6 means that 10^6 (1,000,000) molecules will partition into octanol for each (1) molecule, which will partition into water.
Source: Krieger, R. in *Handbook of Pesticide Toxicology*, Academic Press, 2001. With permission.

that it is only good for compounds that will elicit an easily measurable response. An example of a biological response would be the vasoconstrictor assay in which the blanching effect of one compound is compared to that of a known compound. This method is perhaps more qualitative that quantitative. The best-known use of this method is in comparison of various hydrocortisone products for skin dermatitis (Wester and Maibach, 1997).

36.2.6 *In Vitro* Methodology

In vitro percutaneous absorption is best done with human skin. The skin should be used as soon as possible, and stored in the refrigerator no longer than 7 days. *In vitro* penetration into skin gives results suitable for distinguishing drug formulations, especially in cases where the drug will not partition into reservoir fluid. Material balance in an *in vitro* study design adds to the overall data presentation. *In vivo* verification of skin absorption, preferably in humans, adds relevance to the *in vitro* data. The human skin sample can be kept viable if stored properly in the refrigerator (freezing kills skin viability) and used appropriately (Wester et al., 1998).

Table 36.2 gives the *in vitro* human skin and *in vivo* percutaneous absorption of several chemicals from a variety of vehicles. The *in vitro* absorption is divided into skin content and receptor fluid (either buffered saline or human plasma) accumulation. Generally, receptor fluid accumulation does not agree with *in vivo* percutaneous absorption. The reason for this is lack of solubility in the receptor fluid. In some cases, skin content (see DDT) was a reflection of *in vivo* absorption because the chemical was able to penetrate skin (and lacking solubility, failed to partition into receptor fluid). Chemicals with high log P (octanol:water partition coefficient) will not partition into receptor fluid (Wester and Maibach, 1997, 1999).

36.3 REGIONAL VARIATION IN HUMAN AND ANIMAL PESTICIDE PERCUTANEOUS ABSORPTION

Feldmann and Maibach (1967) first explored the potential for regional variation in percutaneous absorption. The first absorption studies were done with ventral forearm,

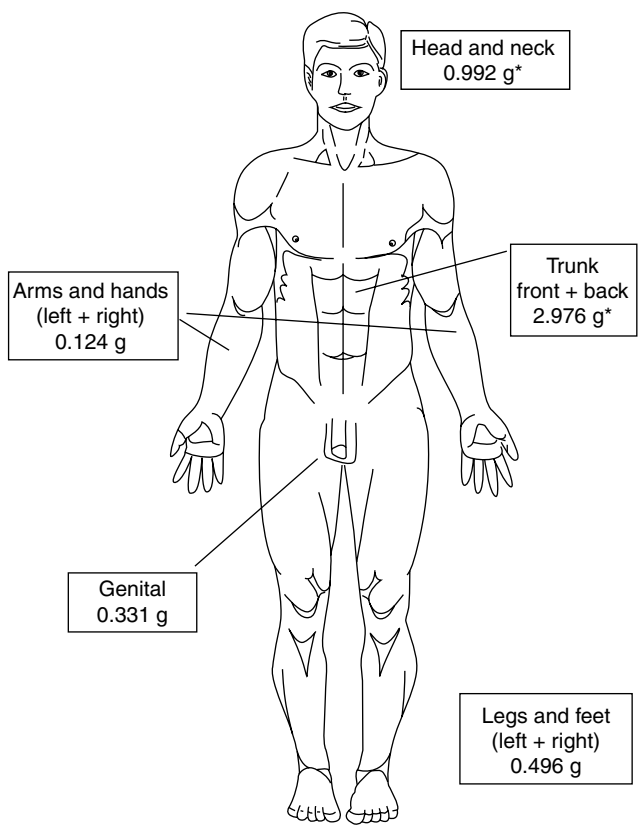


FIGURE 36.2 Different parts of the body vary in percutaneous absorption. This is an important consideration in risk assessment. (Adapted from Krieger, R. *Handbook of Pesticide Toxicology*, Academic Press, 2001. With permission.)

TABLE 36.3
Effect of Anatomical Region on *In Vivo* Percutaneous Absorption of Pesticides in Human

	Percent Dose Absorbed		
Anatomical	**Hydrocortisone**	**Parathion**	**Malathion**
Forearm	1.0	8.6	6.8
Palm	0.8	11.6	5.8
Foot, ball	0.2	13.5	6.8
Abdomen	1.3	18.5	9.4
Hand, Dorsum	–	21.0	12.5
Forehead	7.6	36.3	23.2
Axilla	3.1	64.0	28.7
Jaw Angle	12.2	33.9	69.9
Fossal cubitalis	–	28.4	
Scalp	4.4	32.1	
Ear canal	–	46.6	
Scrotum	36.2	101.6	

Source: Krieger, R. in *Handbook of Pesticide Toxicology*, Academic Press, 2001. With permission.

TABLE 36.4
Site Variation and Decontamination Time for Parathion

Skin Residence Time before Soap-and-Water Wash	**Parathion Dose Absorbed[a] (%)**		
	Arm	**Forehead**	**Palm**
1 min	2.8	8.4	
5 min			6.2
15 min	6.7	7.1	13.6
30 min		12.2	13.3
1 h	8.4	10.5	11.7
4 h	8.0	27.7	7.7
24 h	8.6	36.3	11.8

[a] Each time is a mean for four volunteers. The fact that there were different volunteers at each time point.

Source: Krieger, R. in *Handbook of Pesticide Toxicology*, Academic Press, 2001. With permission.

because this site is convenient to use. However, skin exposure to chemicals exists over the entire body. They first showed regional variation with the absorption of parathion (Figure 36.2). The scrotum was the highest absorbing skin site (scrotal cancer in chimney sweeps is the key). Skin absorption was the lowest for the sole, and highest around the head and face.

Table 36.3 gives the effect of anatomical region on the percutaneous absorption of pesticides in humans (Maibach et al., 1971). There are two major points in this study. First, regional variation was confirmed with the different chemicals; note that parathion and malathion are chemically related to some chemical warfare agents. Second, those skin areas that would be exposed to the pesticides, the head and face, were of the higher absorbing sites. Body areas most exposed to environmental contaminants are among the areas with the higher skin absorption.

Table 36.4 gives site variability for parathion skin absorption with time. Soap and water wash in the first few minutes after exposure is not a perfect decontaminant. Site variation is apparent early in skin exposure (Wester and Maibach, 1985). Decontamination is discussed later.

Guy and Maibach (1985) took the hydrocortisone and pesticide data and constructed penetration indices for five anatomical sites (Table 36.5). The indices might be used with their total surface areas (Table 36.6) when estimating systemic availability relative to body exposure sites.

Van Rooy et al. (1993) applied coal-tar ointment to various skin areas of volunteers and determined absorption of

TABLE 36.5
Penetration Indices for Five Anatomical Sites Assessed Using Hydrocortisone Skin Penetration Data and Pesticide (Malathion and Palathion) Absorption Results

	Penetration Index Based on	
Site	**Hydrocortisone Data**	**Pesticide Data**
Genitals	40	12
Arms	1	1
Legs	0.5	1
Trunk	2.5	3
Head	5	4

Source: Krieger, R. in *Handbook of Pesticide Toxicology*, Academic Press, 2001. With permission.

TABLE 36.6
Body Surface Areas Distributed over Five Anatomical Regions for Adults and Neonate

	Adult		Neonate	
Anatomical Region	**Body Area (%)[a]**	**Area (cm^2)**	**Body Area (%)**	**Area (cm^2)**
Genital	1	180	1	19
Arms	18	3240	19	365
Legs	36	6480	30	576
Trunk	36	6480	31	595
Head	9	1620	19	365
	Total	18,000		1920

[a] Note the "rule of 9" when trying to remember human body surface areas.

Source: Krieger, R. in *Handbook of Pesticide Toxicology*, Academic Press, 2001. With permission.

polycyclic aromatic hydrocarbons (PAH) by surface disappearance of PAH and the excretion of urinary I-OH pyrene. Using PAH disappearance, skin ranking (highest to lowest) was shoulder > forearm > forehead > groin > hand (palmar) > ankle. Using I-OH pyrene excretion, skin ranking (highest to lowest) was neck > calf > forearm > trunk > hand. Table 36.7 compares their results with Guy and Maibach (1985).

Wester et al. (1984) determined the percutaneous absorption of paraquat in humans. Absorption was the same for the leg ($0.29 \pm 0.02\%$), hand ($0.23 \pm 0.1\%$), and forearms ($0.29 \pm 0.1\%$). Here, the chemical nature of the low-absorbing paraquat overcame regional variation.

Skin absorption in rhesus monkey is considered to be relevant to that of humans. Table 36.8 shows the percutaneous absorption of testosterone (Wester et al., 1980), fenitrothion, aminocard, and diethyltoluamide (DEET) (Moody and Franklin, 1987; Moody et al., 1988), in the rhesus monkey compared with the rat. What is interesting is that for the rhesus monkey, there is regional variation between forehead (scalp) and forearm. If one determines the ration of

TABLE 36.7
Absorption Indices of Hydrocortisone and Pesticides (Parathion/Malathion), Calculated by Guy and Maibach (1985), Compared with Absorption of Pyrene and PAH for Different Anatomical Sites by Van Rooy et al. (1993)

	Absorption Index			
Anatomical Site	**Hydrocortisone[a]**	**Pesticides[b]**	**Pyrene[c]**	**PAH[d]**
Genital	40	12	–	–
Arms	1	1	1	1
Hand	1	1	0.8	0.5
Legs/ankle	0.5	1	1.2	0.8/0.5
Trunk/shoulder	2.5	3	1.1	/2.0
Head/neck	5	4	/1.3	1.0

[a] Based on hydrocortisone penetration data (Feldmann and Maibach, 1967).

[b] Based on parathion and malathion absorption data (Maibach et al., 1971).

[c] Based on excreted amount of 1-OH-pyrene in urine after coal-tar ointment application (Van Rooy et al., 1993).

[d] Based on the PAH absorption rate constant (K_a) after coal-tar ointment application (Van Rooy et al., 1993).

Source: Krieger, R. in *Handbook of Pesticide Toxicology*, Academic Press, 2001. With permission.

TABLE 36.8
Percutaneous Absorption of Fenitrothion, Aminocard, DEET, and Testosterone in Rhesus Monkey and Rat

		Applied Dose Absorbed Skin Site		
Chemical	**Species**	**Forehead**	**Forearm**	**Back**
Fenitrothion	Rhesus	49	21	
	Rat			84
Aminocard	Rhesus	74	37	
	Rat			88
Testosterone	Rhesus	20.4[a]	8.8	
	Rat			47.4
DEET	Rhesus	33	14	
	Rat			36

[a] Scalp.

Source: Krieger, R. in *Handbook of Pesticide Toxicology*, Academic Press, 2001. With permission.

forehead (scalp) to forearm for the rhesus monkey and compares the results with human, the results are found to be similar (Table 36.9). Therefore, the rhesus monkey can be a relevant animal model for human skin regional variation.

36.4 PERCUTANEOUS ABSORPTION FROM CHEMICALS IN CLOTHING

Chemicals in cloth cause cutaneous effects. For example, Hatch and Maibach (1986) reported that chemicals added to cloth in 10 finish categories (dye, wrinkle resistance, water repellency,

TABLE 36.9
Percutaneous Absorption Ratio for Scalp and Forehead to Forearm in Humans and Rhesus Monkeys

		Percutaneous Absorption Ratio	
Chemical	**Species**	**Scalp/Forehead**	**Forehead/Forearm**
Hydrocortisone	Human	3.5	6.0
Benzoic acid	Human		2.9
Parathion	Human	3.7	4.2
Malathion	Human		3.4
Testosterone	Rhesus	2.3	
Fenitrothion	Rhesus		2.3
Aminocard	Rhesus		2.0
DEET	Rhesus		2.4

Source: Krieger, R. in *Handbook of Pesticide Toxicology*, Academic Press, 2001. With permission.

TABLE 36.10
***In Vitro* Percutaneous Absorption of Glyphosate and Malathion from Cloth through Human Skin**

Chemical	**Donor Conditions**	**Treatment**	**Percent Dose Absorbed**
Glyphosate	1% Solution (water)	None	1.42 ± 0.25
	1% Solution on cloth	0 h	0.74 ± 0.26
	1% Solution on cloth	24 h	0.08 ± 0.01
	1% Solution on cloth	48 h	0.08 ± 0.01
	1% Solution on cloth	Add water	0.36 ± 0.07
Malathion	1% Solution (water/ethanol)	None	8.77 ± 1.43
	1% Solution on cloth	0 h	3.92 ± 0.49
	1% Solution on cloth	24 h	0.62 ± 0.11
	1% Solution on cloth	48 h	0.60 ± 0.14
	1% Solution on cloth	Add water/ethanol	7.34 ± 0.61

Note: Both glyphosate and malathion in solution (treatment = none) area absorbed through human skin. Glyphosate and malathion on cotton cloth show absorption in skin; depending upon time chemical was added to cloth (treatment = 0, 24, 48 h). When the cloth was wetted (treatment = add water/ethanol), the transfer of glyphosate and malathion from cloth to human skin was increased. This suggests that sweating, skin oil, or even rain may facilitate transfer of chemicals from cloth to skin.

Source: Krieger, R. in *Handbook of Pesticide Toxicology*, Academic Press, 2001. With permission.

soil release, and so on) caused irritant and allergic contact dermatitis, atopic dermatitis exacerbation, and urticarial and phototoxic skin responses. This is qualitative information that chemicals will transfer from cloth to skin *in vivo* in humans. Quantitative data are lacking. Snodgrass (1992) studied permethrin transfer from treated cloth to rabbit skin *in vivo*. Transfer was quantitative but less than expected. Interestingly, permethrin remained within the cloth after detergent laundering.

In other studies (Wester et al., 1996), *in vitro* percutaneous absorption of glyphosate and malathion through human skin were decreased when added to cloth (the cloth then placed on skin), and this absorption decreased as time passed over 48 h (Table 36.10). It is assumed that with time, the chemical will sequester into deep empty spaces of the fabric, or some type of bonding is established between chemical and fabric. When water was added to glyphosate/cloth and water/ethanol to malathion/cloth, the percutaneous absorption increased (malathion to levels from solution). This perhaps reflects clinical situations where dermatitis occurs most frequently in human sweating areas (axilla, crotch). The clothing must not be a collection system for pesticides, and it cannot be assumed that laundry will remove the agents.

Obendorf et al. (2003) conducted a comprehensive study evaluating the different characteristics in clothing that affect pesticide absorption. Starching fabric reduced pesticide transport compared to those without starching while carboxymethylated fabrics showed higher transport of pesticides compared to the synthetic membrane. Bleached and mercerized fabrics showed much lower transport of pesticides compared to the synthetic membrane. Denim fabrics showed a much greater reduction in pesticide transport than shirt-weight fabrics.

36.5 ENHANCERS OF PERCUTANEOUS ABSORPTION OF PESTICIDES

Certain chemicals can actually lead to an enhanced absorption of pesticides. One such chemical that is used frequently among agricultural workers is sunscreen. Brand et al. (2002) showed that six of a total nine tested sunscreens in fact led to an increase in percutaneous absorption of 2,4-dichlorophenoxyacetic acid, a commonly used herbicide, when tested in hairless mice. More recently, Brand et al. (2003) showed that sunscreens containing chemical UV absorbers have enhanced absorption over those that do not contain these absorbers. However, these enhancement properties due to the chemical UV absorbers were mitigated when the researchers used phenyl trimethicone as a solvent.

Feldmann and Maibach studied the effect of stripping and occlusion on penetration of hydrocortisone in human skin. In skin that was stripped or occluded, there was a doubling in amount of hydrocortisone dose recovered in urine over a 10 days period and significantly altered hydrocortisone absorption rate curve. Nielsen (2005) studied this phenomenon in relation to pesticide absorption in slightly damaged skin. In their experiment, they exposed an *in vitro* sample of human skin to sodium lauryl sulfate, a commonly used detergent compound found in many household products and soaps, for 3 h to induce damage to the skin barrier and then exposed the skin to a group of pesticides. They concluded that skin damage affects the rate, lag time, and total penetration of chemicals with the most hydrophilic pesticides being most affected.

With the knowledge that topically applied ethanol is a dermal penetration enhancer, Brand et al. (2004) studied

whether chronic alcohol consumption would also lead to pesticide penetration enhancement. In a study on rats, Brand concluded that regular alcohol consumption led to altered properties of the dermal barrier and led to an increased penetration of pesticides through rat skin. However, this phenomenon has yet to be proven true in humans.

36.6 SKIN DECONTAMINATION

Although decontamination of a chemical from the skin is commonly done by washing with soap and water, as it has been assumed that washing will remove the chemical, recent evidence suggests that many times the skin and the body are unknowingly subjected to enhanced penetration and systemic absorption/toxicity because the decontamination procedure does not work or may actually enhance absorption.

Zendzian (2003) studied the effects of pesticide residue in washed skin and its contribution to dermal toxicity. Nine of 19 pesticides tested in rats showed an increase in systemic concentration after washing indicative of a postwash absorption and increased toxicity. Moody and Maibach (in press) proposes a mechanism for this wash-in effect in that by washing chemicals off of one's skin, some chemicals can actually be washed into the system and become bioavailable through the systemic cutaneous blood supply.

Figure 36.3 illustrates skin decontamination of alachlor with soap and water or water only over a 24 h dosing period, suing grid methodology. Note that the amount recovered decreases over time, which happens because this is an *in vivo* system and percutaneous absorption is taking place, decreasing the amount of chemical on the skin surface. There may also be loss due to skin desquamation. The second observation is that alachlor is more readily removed with soap-and-water wash than with water only. The reason is alachlor is lipid soluble and needs the surfactant system for more successful decontamination (Wester et al., 1991, 1992).

In the preceding illustration, decrease in alachlor wash recovery over time was thought to be due to ongoing absorption and loss due to skin desquamation. These factors are probably true, but probably not the main reason, which is soap-and-water wash effectiveness. In the home and workplace, decontamination of a chemical from skin is traditionally done with a soap-and-water wash, although some workplaces may have emergency showers. It has been assumed that these procedures are effective, yet workplace illness and even death occur from chemical contamination. Water, or soap and water, may not be the most effective means of skin decontamination, particularly for fat-soluble materials. A study was undertaken to help determine whether there are more effective means of removing methylene bisphenyl isocyanate (MDI) from the skin. MDI is an industrial chemical for which skin decontamination, using traditional soap and water and nontraditional polypropylene glycol, a polyglycol-based cleanser (DTAM), and corn oil were all tried *in vivo* on the rhesus monkey, over 8 h (Figure 36.4). Water, alone and with soap (5 and 50% soap), was partially effective in the first hour after exposure, removing 51–69% of the applied dose. However, decontamination fell to 40–52% at 4 h and 29–46% by 8 h. Thus, the majority of MDI was not removed by the traditional soap-and-water wash; skin tape stripping after washing confirmed that MDI was still on the skin. In contrast, polypropylene glycol, DTAM, and corn oil all removed 68–86% of the MDI in the first hour, 74–79% at 4 h, and 72–86% at 8 h. Statistically, polypropylene glycol, DTAM, and corn oil were all better ($p < 0.05$) than soap and water at 4 and 8 h after dose application. These results indicate that a traditional soap-and-water wash and the emergency water shower are relatively ineffective at removing

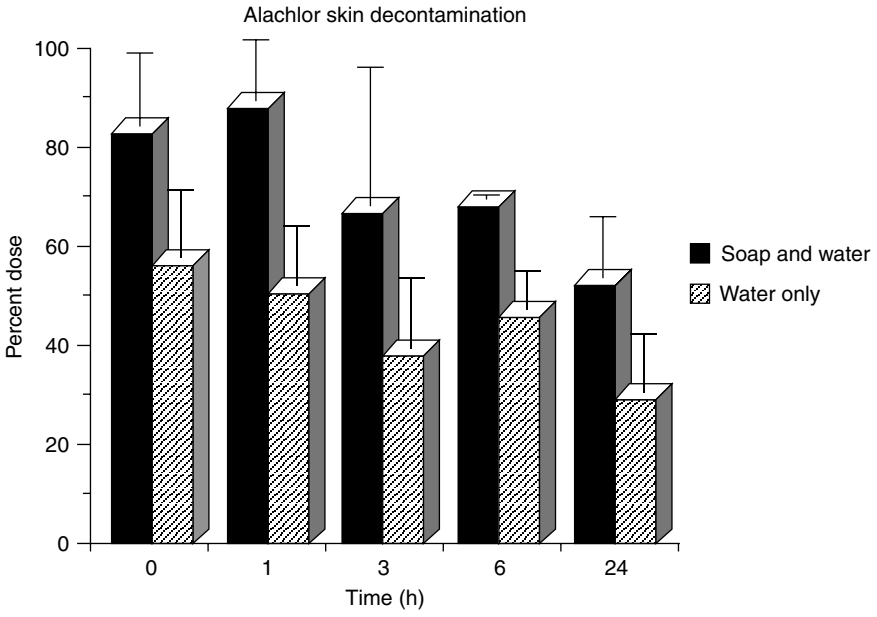


FIGURE 36.3 Alachlor is a lipophilic chemical, which is better removed from skin by soap and water than by water only. (Adapted from Krieger, R. *Handbook of Pesticide Toxicology*, Academic Press, 2001. With permission.)

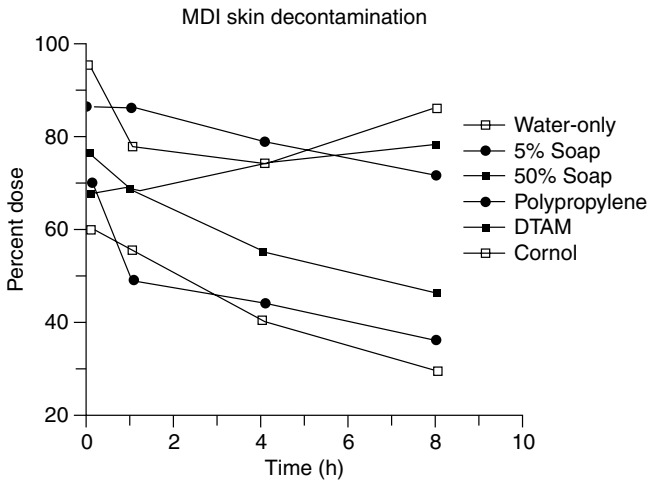


FIGURE 36.4 Mean percent applied dose of MDI removed with designated decontamination procedure at designated time period. Water, and soap and water are the least effective, especially at 4 and 8 h. (Adapted from Krieger, R. *Handbook of Pesticide Toxicology*, Academic Press, 2001. With permission.)

MDI from the skin. More effective decontamination procedures, as shown here, are available. These procedures are consistent with the partial miscibility of MDI in corn oil and polyglycols (Wester et al., 1999). Thus, if there is skin contamination with a pesticide and the skin is washed with soap and water, it cannot be assumed that the pesticide has been removed from the skin.

36.7 DISCUSSION

The lesson partially learned but still ongoing is that pesticide use can achieve its chemically intended goals, but that continued knowledge in human risk assessment needs to be achieved. Understanding percutaneous absorption as a major route of pesticides entering the body is an integral part of the risk assessment process. Data in humans can be achieved safely using trace methodology, and with low-risk doses coupled with high-tech analytical methodology. Data from animal and computer models are simpler to use. Safer is debatable if the models are not validated to man, because the resulting risk assessment may also be wrong.

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37 Tape Stripping Method versus Stratum Corneum

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37.1 INTRODUCTION

Tape stripping is a useful method for removing the stratum corneum (SC) and obtaining more information about function of this thin layer as a main barrier for skin penetration. Typically, an adhesive tape is pressed onto the test site and is subsequently abruptly detached. The number of tape strips need to remove the SC varies with age, sex, and possibly ethnicity (Palenske and Morhenn, 1999).

Tape stripping has been used to various dermatological and pharmaceutical fields to measure the SC mass and thickness (Bashir et al., 2001; Blank et al., 1984; Dreher et al., 1998; Herkenne et al., 2006; Jakasa et al., 2006; Kalia et al., 2001; Schwindt et al., 1998; Weigmann et al., 2005), to investigate percutaneous penetration and disposition of topically applied drug *in vivo* (Benfeldt and Serup, 1999; Benefeldt et al., 1999; Choi et al., 2003; Jacobi et al., 2003; Potard et al., 2000; Rougier et al., 1987), and to disrupt skin barrier function (Benfeldt and Serup, 1999; Benefeldt et al., 1999; Fluhr et al., 2002). Also, this technique has been used to collect SC lipids samples (Weerheim and Ponec, 2001), to detect proteolytic activity associated with the SC (Beisson et al., 2001), and to quantitatively estimate esterase activities in the SC (Mazereeuw-Hautier et al., 2000). Tape stripping is a quantitative and minimally invasive assay for the detection of metal on and in the skin (Cullander et al., 2000; Hostynek et al., 2001, 2006). Tape stripping has been used to disrupt the skin before percutaneous peptide (protein) and DNA immunization (Liu et al., 2001; Seo et al., 2000; Takigawa et al., 2001; Watabe et al., 2001).

Tape stripping is of sufficient utility to have been proposed by the FDA as part of a standard method to evaluate bioequivalence of topical dermatological dosage forms (Ikeda et al., 2005; Loden et al., 2004; Shah et al., 1998; Weigmann et al., 2005). Ikeda et al. (2005) reported the cutaneous bioavailability of topically applied maxacaltol ointment *in vivo* by tape stripping. Tape stripping is simple, inexpensive, and minimally invasive method; it has been the most frequently used method for investigation of the skin penetration, barrier function, and the involvement factors in skin pathologies. In addition, tape stripping is fast and easy to use in human studies.

This chapter reviews the stripping method, considering factors, analytic method of drug in the SC after stripping, and its application on the penetration enhancement into SC and topical vaccination and summarizes recent data.

37.2 STRATUM CORNEUM AND ITS FUNCTIONS

SC is a stratified squamous epithelium lining the body surface that plays an important antidesiccating role as a barrier function and a reservoir for topically applied substances (Pelchrzim et al., 2004). SC consists of nonviable cornified cells (corneocytes) embedded in lipid-rich intercellular domains (intercorneocyte spaces). Intercellular domains comprise free fatty acids (FFA), cholesterol (CHOL), and ceramides (CER), together with smaller amounts of cholesteryl sulfate, sterol, triglycerides, squalene, *n*-alkanes, and phospholipids. Nine different extractable CER have been detected in human SC, which are classified as CER1 to CER9 (Stewart and Downing, 1999; Wertz et al., 1985). The CER can be subdivided into three main groups, based on the nature of their head group architecture (sphingosine, phytosphingosine, or 6-hydroxysphingosine).

SC lipids localize mainly in the intercellular space with little in the corneocytes (Moghimi et al., 1999). These lipids

exist as a continuous lipid phase; occupying about 20% of the SC volume, arranged in multiple lamellar structures. All ceramides and fatty acids found in SC are rod and cylindrical in shape; this physical attribute makes them suitable for the formation of highly ordered gel phase membrane domains. CHOL is capable of either fluidizing membrane domains or of enhancing rigidity, depending on the physical properties of the other lipids and the proportion of CHOL relative to the other component (Wertz, 2000). Intracellular lipids that form the only continuous domain in the SC are required for a major competent barrier.

Efforts have been undertaken to characterize the lipid lamellar regions. X-ray diffraction studies on native SC have demonstrated that the SC lipids are organized in two coexisting crystalline lamellar phases: the short periodicity phase (SPP) with a periodicity of approximately 6 nm and the long periodicity phase (LPP) with a periodicity of approximately 13 nm (Bouwstra et al., 1991, 1995, 2003). The LPP and its predominantly orthorhombic lipid packing are considered to be crucial for the skin barrier function. SC lipids, CER, CHOL, and FFA form the orthorhombic lateral packing, a densely packed structure. However, in equimolar mixtures prepared for CHOL and CER, the major lipid fraction forms a lamellar phase (hexagonal lateral packing) with periodicity of 12.8 nm. Addition of FFA to CER/CHOL mixtures induced a transition from a hexagonal to orthorhombic lateral packing (Bouwstra and Honeywell-Nguyen, 2002). Therefore, the formation of the characteristic LPP depends on the presence of CHOL and specific CER, in particular CER1, whereas FFA are required for the crystalline (orthorhombic) character of the lateral lipid packing (Bouwstra et al., 1998, 2002; McIntosh et al., 1996).

Diseases such as atopic dermatitis, psoriasis, and contact dermatitis are associated with barrier dysfunction. Most skin disorders that have a diminished barrier function present a decrease in total CER content with some differences in their pattern (Choi and Maibach, 2005; Macheleidt et al., 2002; Matsumoto et al., 1999; Okamoto et al., 2003). Pilgram et al. (2001) reported that in case of diseased skin, an impaired barrier function is related to an altered lipid composition and organization. In atopic dermatitis SC, they found that, in comparison with healthy SC, the presence of the hexagonal lattice (gel phase) is increased with respect to the orthorhombic packing (crystalline phase). From lipid composition studies of atopic skin, it has been found that intercellular lipids, especially ceramides, play an important role in the barrier function and lipid organization. Man-Qiang et al. (1993) suggested that for the formation of a component SC barrier, the CER, CHOL, and FFA should be present in an equimolar ratio. Man et al. (1996) reported that three major SC lipids are required for permeability barrier homeostasis and equimolar composition of major lipids is increased up to threefold acceleration of barrier repair. Barrier repair creams including natural components of SC lipids have been used to treat skin disease (Chamlin et al., 2001; Mortensen et al., 2001). Chamlin et al. (2001) reported a phase I trial of a repair cream in childhood atopic dermatitis.

The physiology of SC differs from gender to gender. Only few studies on gender-related differences in skin physiology have been performed and they provided conflicting results (Blank, 1939; Ehlers et al., 2001; Ohman and Vahlquist, 1994; Wilhelm et al., 1991). Jacobi et al. (2005) investigated the effect of gender on the physiology of the SC. The skin of women was characterized by a significantly higher pH value (5.6 ± 0.4) than that of men (4.3 ± 0.4, $p < 0.05$). There were no significant differences between women and men volunteers in TEWL, SC hydration, and sebum content. Protein absorption was the only other parameter significantly dependent on gender. The difference of skin pH and protein absorption might be caused by differences in human biology, such as hormonal status.

37.3 SC REMOVAL METHODS AND EFFECT OF STRIPPING

To remove SC, tape stripping for mechanical removal of corneocytes and solvent-extraction method to remove both polar and nonpolar SC lipids is used. Tape stripping is a useful technique for selectively removing the skin's outermost layer, while solvent extraction is a delipidization process in SC.

In general, a clinical description of the barrier disruption differs depending on the disruption methods. For tape-stripped skin, the typical description was moderate erythema and a glistening surface due to total removal of the SC; for acetone-treated skin, the description was minimal or no erythema and slight superficial dryness; and for chloroform–methanol mixture, the description was deep erythema and edema (Benefeldt et al., 1999). Thus, an organic solvent method using chloroform–methanol mixing may be more aggressive than standard tape.

The change of skin condition after stripping differs depending on stripping (Table 37.1). Fluhr et al. (2002) investigated the barrier recovery pattern after tape stripping or acetone delipidization at five body sites in healthy volunteers. The fastest barrier recovery after tape stripping and acetone was observed on the forehead, followed by the back. But there are differences in SC capacitance values following acetone and tape stripping. In the case of acetone, there were no statistically significant differences in SC capacitance between body sites. In contrast, tape stripping produces significant differences in capacitance values between body sites. The capacitance increases are related to strong barrier damage by tape stripping. However, the decrease of capacitance appears related to lipid extraction. Benefeldt and Serup (1999) reported that salicylic acid penetration was greatly increased with the tape stripping, but not with acetone in the skin of hairless rats.

After barrier disruption, there are typically no adverse effects, such as infection or scaring. However, disruption of permeability barrier by tape stripping induces activation and maturation of epidermal Langerhans' cells (Nishijima et al., 1997). This process is important in inducing immune response *in vivo* and in immunizing with peptide and protein by a percutaneous method.

TABLE 37.1
Physiological Changes of the Human and Rat Skin after Stripping

Type of Skin	Barrier Perturbation	None	Tape Stripping[a]	Acetone[b]
Human	TEWL (g/cm^2/h)[c]	4.3 ± 2.2	30.6 ± 22.2	9.1 ± 7.5
	Erythema (arbitrary unit)[d]	8.7 ± 2.8	11.6 ± 2.8	9.2 ± 1.5
Rat	ΔTEWL[e]	0	69 ± 14	6 ± 3
	ΔErythema[e]	0	2.41 ± 0.87	0.95 ± 1.66

[a] Tape stripping was achieved by applying 2.5 × 5 cm (human) and 5 × 5 cm (rat) piece of Transpore tape with firm pressure and repeating the procedure 20 (human) and 10 (rat) times, respectively.

[b] Acetone was treated by gentle wiping with large cotton buds socked in 100% acetone for 3 min.

[c] Fifteen minutes after barrier perturbation procedures, TEWL was measured using an evaporimeter and recorded in triplicate.

[d] Colorimetry measures skin color by analyzing the light reflected from the skin surface according to the standardization protocol for the content of green-red (a^*) and yellow-blue (b^*) color and skin brightness (L^*). The a^* redness parameter is a measure of a erythema.

[e] TEWL and erythema from the barrier perturbed skin area minus the value from the untreated side.

Source: Benfeldt, Serup, and Menne, Br. J. Dermatol., 140, 739–748, 1999.

37.4 STRIPPING FACTORS

When the tape stripping is employed, the following factors are important: (1) number of strips, (2) types and size of tapes, (3) the pressure applied to the strip prior to stripping and the peeling force applied for removal, and (4) anatomic site. Some parameters are summarized in Table 37.2. We summarize the effect of the type of tape and number of strips on the stripping.

Dreher et al. (1998) improved the method by quantifying the amount of human SC removed by each strip utilizing a colorimetric protein assay. With this method, Bashir et al. (2001) determined the physical and physiological effect of SC tape stripping, utilizing tapes with different physicochemical properties. Three commercial adhesive tapes utilized were D-Squame® (CuDerm, Dallas, Texas), Transpore® (3M, St. Paul, Minnesota, batch no. 2002-12 AP), and Micropore® (3M, St. Paul, Minnesota, batch no. 2001-08 AN). D-Squame is precut into disc shape. Transpore and Micropore are provided as a standard roll. Table 37.3 shows the components of three commercial adhesive tapes and the effect of tapes on the transepidermal water loss (TEWL) depending on the number of strip. Bashir et al. (2001) demonstrated that no significant difference was found in the kinetic parameters (mean water diffusion coefficient, SC thickness, and permeability) between the tapes. However, there are differences in the mean TEWL values. Mean TEWL increased significantly

TABLE 37.2
Comparison to Tape-Stripping Methods

Type of Tape	Stripping Number	Size	Applied Pressure	Time	Study
D-Squame	40	14 mm	10 Kpa	2 s	Bashir et al. (2001)
Transpore	40		10 Kpa	2 s	Bashir et al. (2001)
Micropore	40		10 Kpa	2 s	Bashir et al. (2001)
D-Squame	16	14 mm	80 g/cm^3	5 s	Potard et al. (2000)
Leukoflex	18–20	7.5 cm^2	Soft pressure		Weerheim and Ponec (2001)
3M invisible	7		Controlled condition	10 s	Fernandez et al. (2002)
Adhesif 3M 6204	10	20 cm^2		2 s	Mazereeuw-Hautier and Muller (2000)
Scotch Book tape 845	20		By rubbing six times		Alberti et al. (2001)
Scotch	7		1-kg-pressure		Wissing and Muller (2002)
Scotch 600	2–5	4 cm	By rubbing		Betz et al. (2001)
Blenderm 3M	6	4 cm^2			Couteau et al. (2001)
Transpore	20	12.5 cm^2	Firm pressure		Benfeldt et al. (1999)
Transpore	10	5 × 5 cm			Benfeldt and Serup (1999)
Teasfilm	20	4 cm^2			Fluhr et al. (2002)
D-Squame	20	3.8 cm^2	Uniform pressure	5 s	Dreher et al. (1998)
D-Squame	16	25 mm	0.365 N/cm^2		Chatelain et al. (2003)
D-Squame	25	25 mm	Uniform pressure	5 s	Simonsen et al. (2002)
D-Squame	20	2.2 cm	10 Kpa pressure	2 s	Bashir et al. (2005)
Tesa	20	3.0 cm^2	2-kg-pressure	10 s	Verma et al. (2003)
Tesa	26–28	1.9 cm	15 g–25 g/cm^2	15 s	Lademann et al. (2005)
Cover-Roll tape	5	10 cm^2	Uniform pressure	2 min	Chao and Nvlander French (2004)
Scotch Book tape 845	20	2.0 cm^2	Uniform pressure		Esposito et al. (2005)
					Ricci et al. (2005)

Note: Tape stripping is employed with different adhesive tape, size, number of strips, and the pressure applied to the strip prior to stripping and the peeling force applied for removal.

TABLE 37.3
Components of Three Commercial Tape and Precise TEWL (g/m²/h) Data per Number of Strips for Three Common Tapes at Dorsal Forearm Site

Number of Strips	Type of Tape		
	D-Squame	Transpore	Micropore
Components	Polyacrylate ester, super clear polymer	Iso-octyl acrylate, methyl acrylic acid copolymer	Iso-octyl acrylate, acrylic acid copolymer
Baseline	10.3	8.78	9.37
10	11.23	10.77	8.88
20	14.15	14.12	10.1
30	21.05	21.12	10.4
40	30.33	31.98	13.4

Note: The tape was applied to the test site with forceps and pressed onto the skin with a standardized 10 Kpa pressure for 2 s. The pressure was then removed and the tape was peeled from the skin unidirectionally.

Source: Bashir, Chew, Anigbogu, Dreher, and Maibach, Skin Res. Technol., 7, 40–48, 2005.

as the deeper layers of the SC reached by tape stripping for the D-Squame and Transpore, but not for the Micropore tape. Therefore, D-Squame and Transporore tapes induce a significant increase in the TEWL, while Micropore tape does not (Table 37.3). The value of TEWL differed depending on the tapes and the number of tape strips. Loffler et al. (2004) investigated the influences of stripping procedures (anatomical site, pressure, pressure duration, and tape removal rate) inherent in each stripping protocol on changes in skin physiology. They reported that stripping results were influenced dramatically by all investigated parameters.

The number of tape strips to remove SC differs by investigators and experimental methods such as *in vivo* and *in vitro* assay (Table 37.2). The FDA guideline recommends 10 tape strips after topical application of a substance. Weerheim and Ponec (2001) reported that the average number of tapes *in vivo* could be 18–20 strips. For some individuals, 40 adhesive tape strips, regardless of the type of tapes, do not disrupt the SC barrier to water (Bashir et al., 2001). Thus, we consider the factors such as the types of tape and number of strips when applying this method.

37.5 TAPE STRIPPING VERSUS PERCUTANEOUS ABSORPTION AND PENETRATION

Percutaneous absorption and penetration is a complex physical and physiological process. This process initiates a series of absorption and excretion that are influenced by a numerous factors. Percutaneous absorption of drug depends mainly on the permeability coefficient of the drug, which is affected by drug polarity, molecular size, the vehicle in which the drug is applied, and the skin barrier. Other important factors are application conditions (nonocclusion or occlusion) and skin integrity, which is affected by disease and trauma, body site, and age (Fang et al., 2001; Feldmann and Maibach, 1965, 1967; Morgan et al., 2003; Rougier et al., 1986, 1987, 1999; Wester and Maibach, 1983; White et al., 2002).

The intracellular lipid domain is a major pathway for permeation of most drugs through the SC and also acts as a major barrier for penetration. As a consequence of its hydrophobic nature, the SC barrier allows the penetration of lipid-soluble molecules more readily than water-soluble drugs. Generally, small, nonpolar, lipophilic molecules are the most readily absorbed, while high water solubility confers less percutaneous absorptive capacity through normal skin (Morgan et al., 2003). The way to overcome the properties of the corneal layer is by disrupting it with physical methods (ultrasound, low- and high-voltage electrical pulsing, and stripping) and chemical enhancers.

The tape-stripping method is mainly used to measure drug concentration and its concentration profile across the SC. The SC is progressively removed by serial adhesive tape stripping and consequently, percutaneous absorption and penetration was significantly increased in stripped skin (Table 37.4). Benfeldt and colleagues (Benfeldt et al., 1999; Benfeldt and Serup, 1999) reported that in microdialysis experiment salicylic acid was highly increased in tape-stripped skin in human and hairless rats at 157- and 170-fold, respectively. Morgan et al. (2003) reported that in microdialysis experiment tape stripping increased penciclovir absorption by 1300-fold and acyclovir absorption by 440-fold. Although tape stripping increased the penetration of drugs into the skin, this is not universal (Arima et al., 1998; Moon et al., 1990; Xiong et al., 1996). Physiological and pathological factors affect drug transport across the living human skin. Bos and Meinardi (2000) suggested the 500-Da rule for the skin penetration of chemical compounds and drugs. This size limit may be changed by the skin abnormalities such as atopic dermatitis and disrupted skin.

Abla et al. (2005) reported the effect of iontophoretic current on the acetaminophen and kyotorphin (peptide) delivery across intact and tape-stripped porcine ear skin. Passive permeation of acetaminophen and kyotorphin across intact porcine ear skin was negligible. After removal of the SC, there was a significant increase in passive permeation of acetaminophen ($294 \pm$ nmol/cm²/h) and kyotorphin peptide (98 ± 31 nmol/cm²/h). However, the application of an iontophoretic current across tape-stripped skin did not result in a further increase in acetaminophen (266 ± 71 nmol/cm²/h) and kyotorphin (100 ± 30 nmol/cm²/h). Iontophoretic studies into the transdermal delivery of lidocaine by Sekkar et al. (2004) and tacrine by Hirsch et al. (2005) across intact and tape-stripped skins have also observed the similar result as Abla et al. (2005). From these results, application of iontophoretic current in the impaired skin did not increase transdermal delivery of applied drugs.

In addition to organic drugs, tape striping increased the penetration of biological macromolecules such as peptide and DNA into viable skin (Liu et al., 2001; Seo et al., 2000;

TABLE 37.4
***In Vivo* Drug Penetration Studies in Barrier-Perturbed Skin**

Barrier Perturbation	Species	Drug	Penetration Ratio[a]	Study
None			1	
Tape stripping	Human	Hydrocortisone	2	Feldmann and Maibach (1965)
	Human (occulsion)	Hydrocortisone	32.7	Feldmann and Maibach (1965)
	Human	Low molecular weight Heparin	1	Xiong et al. (1996)
	Human	Methylprednisolone aceponate	91.5	Gunther et al. (1998)
	Human	Salicylic acid	157	Benfeldt et al. (1999)
	Human	Penciclovir	1300	Morgan et al. (2003)
	Human	Aciclovir	440	Morgan et al. (2003)
	Hairless guinea pig	Benzoic acid	2.1	Moon et al. (1990)
	Hairless guinea pig	Hydrocortisone	3	Moon et al. (1990)
	Rat	Nicotinic acid	10.8	Bronaugh and Stewart (1985)
	Rat	Cortisone	2.5	Bronaugh and Stewart (1985)
	Rat	Salicylic acid	0.8–46	Murakami et al. (1998)
	Hairless rat	Oligonucleotide	24–166	Regnier et al. (2000)
	Hairless rat	Salicylic acid	170	Benfeldt and Serup (1999)
	Hairless mouse	Nitroglycerin	9.0	Higo et al. (1992)
	Hairless mouse	Enoxacin	7.5	Fang et al. (2001)
	Hairless mouse	Biphenylacetic acid	1	Arima et al. (1998)
	Porcine ear	Acetaminophen	290	Abla et al. (2005)
	Porcine ear	Kyotorphin	100	Abla et al. (2005)

[a] Penetration ratio varies among drugs and species investigated. Most of the studies used traditional radiolabeling and HPLC techniques. In case of salicylic acid, the study defined the cutaneous penetration and systemic absorption during 20 min intervals over a period of 4 h after drug administration.

Takigawa et al., 2001; Watabe et al., 2001). Topically applied oligonucleotides (ONs) and DNA do not penetrate normal human SC. But removal of SC by tape stripping led to extensive penetration of ONs and DNA throughout the epidermis. Regnier et al. (2000) compared ONs penetration through intact and stripped hairless rat skin. Stripping increased ONs concentration by 1 or 2 orders of magnitude (24- to 166-fold increase) (Table 37.4). In case of plasmid DNA, Yu et al. (1999) reported that transfer gene activity depends on the number of stripping. They applied a cytomegalovirus-chloramphenicol acetyltransferase expression plasmid to stripped area and found that the transfer gene expression was higher in the murine skin samples stripped five times prior to DNA application compared with those stripped three times prior to DNA application. This result indicated that abrasion of the skin prior to DNA application could improve cutaneous gene transfer and expression. Taken together, tape stripping is commonly used to enhance the delivery of chemical drugs and biological macromolecules.

The determination of penetration pathways of topically applied drugs into the skin has been well evaluated. However, a direct and noninvasive quantification of the amount of topically applied drugs penetrated into the hair follicular had not been available. Teichmann et al. (2005) reported differential stripping method to determine the amount of topically applied drug penetrated into the hair follicles. They used differential stripping techniques with a tape stripping and a cyanoacrylate skin surface biopsy. Tape stripping was used to remove the part of the SC that contained the topically applied dye. Subsequently, the follicular contents were ripped off by cyanoacrylate skin surface biopsy. Differential stripping technique is a new method that can be used to study the penetration of topically applied substances into the hair follicular infundibula noninvasively and selectively.

37.6 TAPE STRIPPING VERSUS ANALYTIC METHODS

To determine the drug concentration and profile into SC, analytical techniques are important. These techniques include skin extraction measurement, horizontal stripping and sectioning, quantitative autoradiography, mass spectrometry, optical microscopy, and spectroscopic methods. Penetration into SC is determined by tape stripping followed by skin extraction and spectroscopic methods. These methods are widely used in determination of drug concentration within skin. Skin extraction is necessary to extract the drug with a suitable solvent and then an appropriate, sensitive analytical such as high-performance lipid chromatography (HPLC), spectroscopy, and scintillation counting is used to quantify the extracted drug. The improving sensitivity of optical instrument has permitted the quantification of drugs in skin by spectroscopic methods. These methods are noninvasive and offer real-time data on penetrated drug localization. These techniques include attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectroscopy, fluorescence

TABLE 37.5
Description of the Techniques Available for Quantifying Drugs in the Skin

Technique	Penetrants Detected	Measuring Depth	Cost	Speed	Complementary Strategies
Skin extraction	Any	All strata	Inexpensive	Rapid	Separation of skin tissue Qualitative autoradiography
Horizontal sectioning	Any	All strata	Inexpensive	Rapid	Separation of follicles Use of follicle-free skin
Quantitative autoradiography	Radiolabelled only	All strata	Expensive	Slow	None
ATR-FTIR spectroscopy	IR-absorbing only	SC	Expensive	Very rapid	Tape stripping
Direct fluorescence spectroscopy	Self-fluorescent only	All strata	Medium	Rapid	Separation of follicles Quantitative fluorescent microscopy
Indirect fluorescence spectroscopy	UV-absorbing only	SC	Medium	Rapid	None
Remittance spectroscopy	UV-absorbing only	SC	Medium	Rapid	None
Photothermal spectroscopy	Strong UV-absorbing	SC	Medium	Rapid	None
Spectroscopy	UV/Visible	SC	Inexpensive	Rapid	Tape stripping
Microscopy	Common or laser	SC	Medium	Rapid	Tape stripping
Mass spectrometry	Metal	SC	Expensive	Rapid	Tape stripping

Source: Hostynek, Dreher, Nakada, Schwindt, Anigbogu, and Maibach, *Acta Derm. Venereol*., 212, 11–18, 2001; Lindemann, Weigmann, Schanzer, Richer, and Audring, *J. Biomed. Opt*., 8, 601–607, 2003; Touitou, Meiden, and Horwiwitz, *J. Control. Release*, 56, 7–21, 1998.

spectroscopy, remittance spectroscopy, confocal microscopy (laser-scanning microscopy), mass spectrometry, and photothermal spectroscopy (Hostynek et al., 2001; Lindemann et al., 2003; Pelchrzim et al., 2004; Touitou et al., 1998). Table 37.5 shows the characterization of the analysis method of drugs in the skin.

Tape stripping and optical spectroscopy are used as a suitable combined method to determine the honey layer profile (Ikeda et al., 2005; Lademann et al., 2005; Lindemann et al., 2003; Pelchrzim et al., 2004; Weigmann et al., 1999, 2001). The application of tape stripping in combination with analytical instruments (mass spectrometry, UV/VIS spectroscopy, microscopy) is checked to determine the local position of topically applied substances inside the SC, the penetration profile (Ikeda et al., 2005; Pelchrzim et al., 2004; Weigmann et al., 2005). The combined use of these analytical methods can test the validity of the dermatopharmacokinetic (DPK) method to assess bioequivalence and bioavailability of topical dermatological drugs.

In addition to drug detection methods, many methods detect metal into and on the skin: inductively coupled plasma-atomic emission spectroscopy (ICP-AES), inductively coupled plasma-mass spectrometry (ICP-MS), atomic absorption spectrometry (AAS), and particle-induced x-ray emission (PIXE) are widely used. ICP-AES permits detection of metals at the trace amount level, obviating the use of radioisotopes. ICP-MS is a technique applicable to microgram per liter (ppb) concentration of several elements in aqueous medium upon appropriate sample preparation of biological materials. AAS is the reference method accepted by the International Union of Pure and Applied Chemistry for trace element analysis. PIXE analysis with a proton microprobe allows the determination of trace elements in epidermal strata prepared by cryosection (Hostynek et al., 2002).

37.7 TAPE STRIPPING VERSUS TOPICAL VACCINATION

Why is the skin a major target for topical vaccination? The skin, an active immune surveillance site, is rich in potent antigen-presenting dendritic cells (DC) such as Langerhans' cells (LC) in the epidermis. LC plays a key role in the immune response to antigenic materials. Skin accessibility makes it an easy target for vaccination. Thus, skin is an attractive target site for topical vaccination and has become the focus of intense study for the induction of antigen-specific immune responses (Babiuk et al., 2000; Choi et al., 2006; Choi and Maibach, 2003; Godefroy et al., 2005). Wang et al. (1996) observed that protein penetrates SC barrier following occlusion by patch application, but immune responses generated in this way are Th2-predominant. This immune response does not elicit cytotoxic T lymphocytes (CTL) response that is important in preventing and therapy against viral infections and tumors.

In addition to disruption of the epidermal barrier, stripping enhances *in vitro* the T-cell-mediated immune response (Nishijima et al., 1997). Tape stripping is immunostimulatory and results in the production and release of IL-1α, IL-1β, TNF-α, IL-8, Il-10, and INF-γ (Nickoloff and Naidu, 1994; Nishijima et al., 1997; Takigawa et al., 2001). Skin barrier disruption by tape stripping also increases co-stimulatory molecule expression (CD86, CD54, CD40, and MHC class II) and the antigen-presenting capacity of epidermal DCs (Kahlon et al., 2003; Takigawa et al., 2001). In addition, tape-stripping facilitates the generation of Th1 immune responses and stimulates LCs migration to cutaneous lymph nodes (Kahlon et al., 2003).

Seo et al. (2000) reported that topical application of tumor-associated peptide onto the SC barrier disrupted by tape stripping in mice induces a protective antitumor response *in vivo*

and *in vitro*. They investigated induction of CTL response on tape-stripped earlobes of C57/BL6 mice by application of CTL epitope peptide onto the SC. The optimal condition for a CTL response was observed 12 and 24 h after tape stripping at peptide doses of 48 and 96 μg per mouse. However, CTL induction was virtually absent when peptide was applied to intact skin (Table 37.6). Kahlon et al. (2003) reported optimization of topical vaccination for the induction of CTL with peptide and protein antigens. They found that tape stripping significantly enhanced antigen-specific antibody (protein) and CTL responses (peptide and protein) measured at 3 and 2 weeks following immunization, respectively (Table 37.6). Stripping resulted in prolonged CTL responses at least 2 months after single immunization. Godefroy et al. (2005) reported the systemic and mucosal antibody responses to protein after its application onto the intact or tape-stripped skin. Application of protein antigen alone onto the intact or tape-stripped skin did not elicit any detectable antibody response. These results are inconsistent with Kahlon et al. (2003). However, when cholera toxin was used as an adjuvant good antigen-specific responses (systemic and mucosal) were measured in mice immunized with protein applied onto the tape-striped skin but not onto the intact skin. These results suggest that stripping can be widely used in inducing immune responses with topical vaccination *in vivo*.

TABLE 37.6
Comparison to CTL Activity of Peptide, Protein, and DNA Immunization with and without Stripping

Antigen	Immunization	Specific Lysis (%)
Peptide	Intact skin	11.0
	Stripped skin[a]	80.0
	Stripped skin + cholera toxin[b]	70.0
Protein[c]	Intact skin	8.0
	Stripped skin	46.0
DNA[d]	Intact Skin	12.7
	Stripped skin	37.0

[a] Cervical lymph node cells (effectors) obtained from mice immunized 10 days earlier with tyrosinase-related protein 2 peptide (VYDFFVWL, 96 μg per mouse) either through intact earlobes or earlobes tape stripped 12 hr earlier were subjected to CTL assay using Lkb target cells pulsed with tyrosinase peptide. CTL assays were performed at effector-to-target ratio of 10.

[b] C57BL/6 mice were immunized on the ear with 25 μg ovalbumin peptide (SIYRYYGL) and 25 μg cholera toxin following tape stripping. Mice were boosted in similar fashion at 1 week and sacrificed at 2 weeks. Ovalbumin expressing EG7 cells were used as target and CTL assays were performed at effector-to-target ratio of 50. The ear skin on the dorsal and ventral side was tape-stripped 10 times (using Scotch Brand 3710 adhesive tape).

[c] C57BL/6 mice were immunized on the ear with 250 μg ovalbumin protein and 25 μg cholera toxin following tape stripping. Mice were boosted in similar fashion at 1 week and sacrificed at 2 weeks. Ovalbumin expressing EG7 cells were used as target and CTL assays were performed at effector-to-target ratio of 50. The ear skin on the dorsal and ventral side was tape-stripped 10 times (using Scotch Brand 3710 adhesive tape).

[d] BALB/c mice were immunized with plasmid DNA coded influenza M protein. Lymphoid cells from each immunized group were restimulated for 5 days using influenza M peptide-pulsed syngenic spleen cells. The peptide pulsed p815 cells were used as targets. CTL assays were performed at effector-to-target ratio of 80. Fast-acting adhesive glue (Alon Alfa®) was smeared on a glass slide to cover the mouse. After an interval of 20–30 s, the slide was ripped off.

In addition to peptide and protein antigen, tape stripping increased the humoral and cellular immune responses of topical DNA antigens (Liu et al., 2001; Watabe et al., 2001). Comparing the immune response with and without stripping, topical application without stripping induced weak antibody response and did not elicit a sufficient CTL response. In contrast, topical application of this vaccine with stripping induced strong antibody responses and elicited substantial CTL responses. There was a significant difference between the results of topical application with and without stripping (Choi et al., 2006; Choi and Maibach, 2003; Liu et al., 2001).

To confirm the protective effect of topical vaccination, Watabe et al. (2001) and Seo et al. (2000) used an influenza and melanoma mouse model, respectively. Watabe et al. (2001) investigated the efficacy of a topical DNA vaccine that expressed the matrix gene of the influenza virus using a mouse model. They topically applied plasmid DNA onto the stripped skin on days 0, 7, and 14. After the third immunization, the mice were challenged with $5LD_{50}$ of influenza virus; 13 of 20 mice (65%) survived when they were topically immunized with plasmid DNA that expressed the matrix gene. When the mice were immunized with inactivated virus topically, only 18% of mice were protected and all mice were dead 7 days after virus inoculation in case of unimmunized control group. These results suggest that the topical administration of DNA vaccine induces a protective immunity against influenza challenge. Seo et al. (2000) investigated the efficacy of topical peptide vaccination for tumor immunotherapy. Mice were immunized twice with tumor-associated peptide at barrier-disrupted skin and were challenged with B16 melanoma tumor cells. B16 tumor cells were virtually completely rejected after epitope peptide immunization via a disrupted barrier. Also, when tumor-bearing mice were treated with epitope peptide on tape-stripped skin, tumor cells regressed with peptide application, and 100% of the mice survived for 1 month and 95% for over 60 days. However, mice treated with peptide application to intact skin died after 34 days. Thus, topical immunization provides a simple, nonadjuvant system, and noninvasive means of inducing potent antitumor immunity that may be exploited for cancer immunotherapy in human.

37.8 UNANSWERED QUESTION AND CONCERNS TO TAPE STRIPPING

Surber et al. (1999) reviewed the tape-stripping technique as standardized tape-stripping technique; many factors remain to be investigated. As shown in Table 37.2, the types and sizes of tapes utilized equally affect the method and the pressure

applied to the strip prior to stripping. A proposed FDA guideline describes serial tape stripping to determine the amount of drug within the skin. By the guidelines, the first tape strip is discarded and the drug is extracted from the remaining pooled strips and the quantified amount is expressed as a mass per unit area. From the guidelines, it is impossible to express the amount of drug substance per unit mass of SC and to determine the proportion of the SC that has been sampled by the tape-stripping method. Although tape stripping is relatively simple to execute, there are many opportunities for experimental artifacts to develop. Tape-stripping samples have a high surface-to-volume ratio, and losses by evaporation can be significant even for chemicals with relatively low volatility. In addition, the tape-stripping experiment is unsuitable for volatile chemicals (Reddy et al., 2002). Considering the current application and convenient of tape-stripping method, topical vaccination and clinical trials for the determination of bioequivalence of topical dermatological products could be improved by stripping standardization.

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38 Parameters Influencing Stratum Corneum Removal by Tape Stripping

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The stratum corneum (SC), the outermost layer of the epidermis, is the main barrier against xenobiotics and protects the body against dehydration. This several micrometer thin skin layer can be studied using a technique known as adhesive tape stripping. Tape stripping is a relatively less invasive method to remove the SC, either partially or entirely.

After its first mention by Pinkus,[1] tape stripping has become a frequently used method in many areas of research.[2] Tape stripping can be used to obtain a more susceptible skin, e.g., prior to the application of an irritant[3] or an allergen.[4–6] Similarly, tape stripping can be performed to induce a defined disruption of the (water) barrier, e.g., to evaluate the effect of a subsequently applied skin care product in barrier restoration.[7] It may be also used to obtain cells for mycological culture[8] or to investigate SC quality.[9] Moreover, it can be used to study skin's barrier function including the uptake of topically administered compounds into the SC.[7,10] Further, the tape stripping technique is currently considered as being a valuable method to assess bioequivalence of topical drug products.[11–14]

Bioengineering methods, such as the measurement of transepidermal water loss (TEWL), can be used to measure changes in barrier properties after tape stripping. To quantify the amount of SC removed by stripping, several methods can be used. Besides weighing of the tape before and after tape stripping,[15] spectroscopic analysis of the tape[16–18] or SC protein extraction from the tape followed by quantification using total protein assay[19,20] were proposed.

Tape stripping appears simple and easy to perform.[21,22] However, there are many parameters influencing the outcome. Various types of tapes exist. They differ in adhesive properties, composition, shape, surface area, and flexibility, indicating that the influence of the tape on the outcome seems apparent.[19,23] Besides adhesive properties, parameters which influence the SC removal can be subsumed in SC's intrinsic characteristics (anatomic site, skin condition, etc.),[24] the pressure with which the tape is applied onto the skin, the duration of pressure and its removal process, as well as other extrinsic factors.[25]

38.1 EXTRINSIC PARAMETERS INFLUENCING TAPE STRIPPING TEST

38.1.1 Anatomic Sites

When comparing changes in TEWL using a standardized tape stripping procedure on different skin sites, the forehead and the back showed a significant higher TEWL increase after removing the same number of strips as compared to the forearm.[25] This may be due to different reasons. The SC of face and trunk is less thick and is composed of less corneocyte layers than the forearm SC.[26,27] Since an equal number of tape strips removes a larger part of SC in face and trunk, tape stripping leads to a faster TEWL increase in these areas as compared to the thicker SC of the forearm.

Furthermore, there are site-dependent differences in spontaneous desquamation[28] and, therefore, probably also SC cohesion, which may additionally explain why the same number of tape strips does not necessarily result in the same TEWL change. The mechanism of spontaneous desquamation is far from completely elucidated, but is believed to depend on physiological and probably also anatomic site-dependent mechanisms, such as corneodesmosome physiology[29] including its proteolysis[30] as well as the composition and structure of the intercorneocyte lipids.[31,32]

A further parameter that may influence the anatomic dependency of SC removal is the pressure resistance when applying the tape, which is likely a function of the biomechanical properties or viscoelasticity of the directly underlying tissue. This seems to be particularly relevant for the forehead where the skull bone is just underneath the skin surface, which may affect the overall pressure on the tape. In what respect the biomechanical properties of the skin and

its underlying tissue influence SC removal by tape stripping remains, however, to be further investigated.

Consequently, tape stripping results obtained from different anatomic regions are not necessarily directly comparable and should be related to the amount of SC removed or, better, normalized with respect to the fraction of SC thickness removed.

38.1.2 Pressure and Contact Time

A longer pressure application period (e.g., from 2 to 10 s) led to an increased TEWL after a significantly smaller number of tape strips, indicating that a larger amount or fraction of SC has been removed.[33] Pressure-sensitive adhesives form a strong adhesive bond with substrates under application of a slight external pressure over a short time.[34,35] This property is known as "tack." As known for such adhesives, tack increases with contact time and pressure up to a plateau corresponding to the so-called critical contact time and contact pressure.[36–38] The critical parameters depend on the adhesive properties as well as the substrate properties. This may be related to the fact that the adhesive glue requires some time to close the gaps between tape and SC surface, leading to an increasing or improved contact area between tape and SC surface.[36]

Similarly, as for contact time, a higher pressure results in a higher tack of pressure sensitive adhesives.[36,37] Hence, as long as the critical contact pressure and time[37,38] is not reached, the amount of SC removed increases with the applied pressure and the period of applied pressure, which results in a corresponding barrier perturbation.

Interestingly, the type of pressure seems important when performing tape stripping since it may influence the homogeneous removal of SC layer. The existence of skin furrows is particularly discussed to influence the outcome of tape stripping.[39,40] Lademann and colleagues showed that the influence of furrows may be minimized when using a standardized roller to apply the tape onto the skin.[39,41] By using this roller the skin is stretched, which flattens the SC and allows, therefore, a more homogeneous SC removal.

38.1.3 Velocity of Removal

In addition to contact time and pressure, the velocity of tape removal was shown to further influence SC mass removed and, thus, barrier perturbation. When strips are removed in a rapid movement, a less pronounced increase of TEWL was observed.[33] In contrast, when the tape removal was performed with a slow movement, more corneocytes seem to adhere to the tape since the barrier was disturbed significantly more with fewer strips.[33] For a given adhesive, the force to remove a tape after application is influenced by the viscoelastic properties of its substrate.[37,38,42] Similarly as for medical adhesives,[43] the skin may become more extended during slow tape removal as compared to fast removal. It is possible that this extension may result in a more efficient SC removal per single tape strip probably due to the fact that corneodesmosomes, which are predominantly responsible for SC cohesion,[29] become more "stressed" during this removal procedure. This hypothesis remains to be studied in detail.

38.1.4 Type of Tapes

When comparing different tape brands, different brands removed differing amounts of SC under standardized conditions.[19] As previously mentioned, this can be explained by the different adhesive characteristics of the tapes.[44] Therefore, it is of importance to define the type of tape before performing a tape stripping test.

In conclusion, numerous parameters significantly affect SC removal when performing tape stripping. Hence, it is recommended that the most relevant tape stripping parameters (tape brand, pressure, time of pressure, removal process) are defined and controlled when a tape stripping study is performed. A definition of the procedure, as can be found in numerous scientific references, like "after application with a moderate pressure over a few seconds onto the skin, the tapes were removed," is not sufficient because the crucial parameter directly influencing SC removal has not been adequately defined or described.

Combining the different procedures in a single test protocol might represent a new dynamic SC stress test (DSCST). For instance, a DSCST may allow distinguishing small differences in intrinsic SC cohesion. Furthermore, DSCST may be a valuable test to investigate in a more dynamic way the effect of topically applied agents such as keratolytics, which influence the SC cohesion.

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39 Quantification of Stratum Corneum Removed by Tape Stripping

Frank Dreher

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39.1 INTRODUCTION

The outermost skin layer, the stratum corneum (SC), can be removed sequentially by repeated application of appropriate adhesive tapes [1]. This technique, commonly known as "SC tape stripping," is a relatively noninvasive frequently used method to investigate the structure, properties, and functions of SC *in vivo* [2]. SC consists of corneocytes embedded in lipid layers and represents the main barrier for skin penetration of xenobiotics. Its thickness in healthy adults varies from 5 to 20 μm, except in the SC of the palm and sole, where SC is thicker. Other techniques to remove SC include skin surface biopsy using cyanoacrylate strips and diverse skin scraping techniques.

Since SC is known to be a reservoir for topically applied chemicals [3,4], its removal by tape stripping has provided useful data on their penetration into skin [5]. Tape stripping is therefore regarded as a valuable method to evaluate cutaneous bioavailability of topically applied chemicals, which may also provide helpful information to understand their dermatotoxicology profile. The possibility to use tape stripping as an alternative method to assess bioequivalence of certain topical drugs is still being debated [6]. Further, tape stripping can be successfully used to investigate intercorneocyte cohesion within the SC [7,8].

This chapter deals with the experimental procedure of tape stripping technique and reviews methods to quantify SC removal.

39.2 TAPE STRIPPING TECHNIQUE

39.2.1 Experimental Procedure

SC tape stripping is carried out by pressing an adhesive tape onto the skin surface, and further removing it by tearing off. The tape is placed to a previously delineated skin surface area according to a standardized procedure by applying a constant pressure (e.g., 100 g cm^{-2}), using a weight or spring system, over an appropriate time period (e.g., 5 s). Then the tape is removed with a single continuous motion. The application and removal procedure may be repeated up to more than 100 times at the same site. Commonly used tapes for skin tape stripping are stationary tapes (e.g., Tesa Film No. 5529, Beiersdorf, Germany), medical tapes (e.g., Transpore®, 3M Co., United States), or for such a purpose specially designed tapes (e.g., D-Squame®, CuDerm Inc., United States). Tapes differ in shape, size, composition, and adhesive properties. Following tape stripping, the solute contained in the SC can be extracted and measured using appropriate analytical methods such as HPLC.

39.2.2 Parameters Influencing SC Removal

Numerous parameters influence the amount of SC removed by a single tape strip. Differences in adhesive properties between [9] as well as within tape brands may result in different amounts of SC removed per surface unit. Pressure [10], time course between application and removal [11], as well

as velocity of tape removal process [10] also significantly influence the SC amount removed. Additionally, SC removal depends on intrinsic skin properties related to race, sex, age, skin site, as well as skin condition (e.g., moistened versus dry, healthy versus pathologic). Moreover, it has been recognized that the SC amount removed by tape stripping varies according to the depth. In general, the first strips remove the largest amounts of SC, because they remove the loosely packed squamous cells. Decreasing amounts of SC are removed with increasing strips due to the fact that the cohesiveness between corneocytes increases with depth [7]. In addition, product application prior to tape stripping should also be considered as a factor influencing the amount of SC removed by sequential tape stripping [12]. For instance, the vehicle components may alter both adhesive properties of the tape; at least for the very first strips, as well as cohesiveness between corneocytes.

39.2.3 Quantification of SC Removal

As described earlier, the amount of SC removed by tape stripping is highly variable and depends on the way the tape stripping procedure is performed, as well as on SC characteristics and conditions. As a consequence, the amount of SC removed by tape stripping is not proportional to the number of strips removed. The SC removal can be determined using various methods including weighing, protein quantification, and spectroscopy.

39.2.3.1 Weighing

Currently, weighing is commonly used to measure the amount of SC removed on a tape strip [13]. Thereby tapes are weighted before and after stripping and the amount of SC is given by weight difference. High precision balances are needed since a very low amount of SC is removed per square centimeter of tape. However, weighing is time consuming and may be biased by water absorption or desorption during weighing procedure before and after stripping [13]. Furthermore, after topical product application, the weighing of SC is only reliable to some extent since the tape strips may also contain applied vehicle and solute.

39.2.3.2 Protein Quantification

As an alternative to weighing, a simple colorimetric method based on commercially available protein assays was recently proposed [14]. Briefly, the total protein assay (e.g., according to Lowry or Bradford) was carried out after immersing the SC containing tapes in a one molar sodium hydroxide solution to extract the soluble SC protein fraction (SC is mainly composed of corneocytes filled with keratins) and neutralizing the solution with one molar hydrochloric acid. The neutralization was realized since most protein assays are not compatible with acidic conditions. Thereby the extracted SC proteins, or their hydrolysate, remain in solution. This quantification method makes it possible to determine accurately and reproducibly as little as a few micrograms of SC adhering to a single tape strip. Furthermore, with the exception of protein-containing products and some other compounds interfering with protein assay, the uptake of product ingredients into the SC after topical application does not interfere with this colorimetric method. In addition, water content of the SC tape strip has no influence on the colorimetric assay. Besides the D-Squame tape, other tapes can be used and are compatible with this assay. Besides performing the protein extraction with each tape strip, the extraction can also be performed with pooled strips such as with five strips [15]. In case it is sufficient to obtain data on pooled strips, this recently described procedure allows in significantly shortening the time of analysis. Alternatively, the protein assay can also be realized in 96 well microplates [16].

When performing bioavailability studies, the discussed method is particularly adapted for hydrophilic solutes, which are chemically stable under alkaline conditions (e.g., hydroxy acids). They can be easily extracted from the SC adhering to tape strips and may be analyzed in parallel. However, this method seems less suitable for hydrophobic and for compounds not stable under the conditions of SC extraction. In that case, tape strips can be divided into two parts: one part can be used for SC protein determination and the other for solute analysis.

A method whereby the SC proteins were directly stained with comassie brilliant blue on the tape without prior SC extraction followed by spectroscopic measurement of the colored tape was unsuccessful [13]. The results were shown to be variable, particularly since the absorbance of colored SC proteins is negligible as compared to light scattering of the SC material adhering to tape strips.

39.2.3.3 Spectroscopic Methods

A few years ago, a method based on the measure of UV/VIS-absorbance was reported to determine the SC amount adhering on tape strips [17]. Unlike methods through protein quantification, this technique does not require any preceding treatment of the SC. As a consequence, the entire tape strip can be used for subsequent analysis including solute extraction. Thereby SC determination is performed at 430 nm directly on the tape (Tesa Film no. 5529) using a double-beam UV/VIS-spectrophotometer, modified to obtain a 1×1 cm^2 light beam. The reference beam chamber contains an unused tape. The absorbance at this wavelength originates from light reflection, scattering, and diffraction by corneocyte aggregates on the tape and was reported to be directly related to SC weight removed by tape stripping. However, chemicals absorbing in the wavelength range of corneocyte absorbance at 430 nm may interfere with the measurement. Further, it remains to be verified whether potential changes in optical properties of corneocytes after topical product application (e.g., due to uptake of water) influence the accuracy of this spectroscopic measurement.

Recently, another spectroscopic (densitometric) method was described [18]. This method measures SC absorption at 850 nm with an instrument developed specifically for this

purpose (SquameScan™ 850A, Heiland Electronic GmbH, Germany). Absorption data were shown to correlate with protein content on the tape, which emphasizes the validity of this approach. Similar to the method measuring SC removal at 430 nm, it remains to be verified whether potential changes in optical (densitometric) properties of corneocytes after topical product application influence the accuracy of this measurement or not.

39.3 SUMMARY AND CONCLUSION

The application of tape stripping technique is well established in dermatopharmacological research, and the technique is appreciated as one of the most useful methods to remove SC allowing investigation of its structure, properties, and functions. However, despite apparent simplicity, the tape stripping technique entails several technical problems and care has to be taken to avoid misleading conclusions when interpreting data. For instance, results given as a function of tape strip number or pooled tape strips have to be interpreted with care, since the amount of SC removed by tape stripping may be highly variable and may depend on numerous factors related to tape stripping procedure and SC properties. Therefore, SC removal by tape stripping should be determined using accurate and reliable methods. Today, weighing still seems to be the most commonly used method for such a purpose. But, due to possible artifacts associated with weighing procedure, SC amounts removed by tape stripping may be more accurately determined through protein quantification of extracted SC proteins or spectroscopic (or densitometric) methods. The currently described alternative methods of weighing require, however, further validation before considering that they indeed provide an adequate and more accurate measure for SC removal by tape stripping.

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40 Isolated Perfused Porcine Skin Flap

Jim E. Riviere

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40.1 INTRODUCTION

There are numerous methods that have been employed to assess the percutaneous absorption of toxic chemicals using both *in vitro* and *in vivo* animal models. There is little debate that *in vivo* human studies are optimal for predicting the absorption of topically applied chemicals in man. However, for highly toxic or carcinogenic chemicals, ethics preclude conducting such studies when a risk analysis is to be conducted. Similar considerations apply to the humane use of animal surrogates. For chemicals that pose little direct adverse risk to man or animals, experimental design and sampling limitations often apply to any *in vivo* study. An important limitation is the inability to noninvasively sample the venous drainage of a topical application site to determine the true cutaneous flux for use as an input into systemic risk assessment models. Although microdialysis provides one approach to this dilemma, it does not allow recovery of all absorbed compound since the dermal vasculature is still intact. Similarly, extensive biopsies may not be taken to quantitate subtle, preclinical morphological or biochemical manifestations of dermatotoxicity.

The next alternative in the hierarchy of model systems would be *in vitro* diffusion cell studies using human skin. Although in most cases these methods may appear to be preferred, there are limitations that may seriously detract from their usefulness. These include studies where vasoactive compounds are being used or where the magnitude or distribution of cutaneous blood flow would affect the subsequent rate and extent of compound absorption or pattern of cutaneous distribution. Vascular changes could result from compound-induced cutaneous irritation where released inflammatory mediators could directly modulate vascular physiology. Some *in vitro* models are not optimal for studying the kinetics of cutaneous metabolism. Another problem is availability of disease-free, fresh human skin from the same individual and body region. Variability in tissue sources may introduce an unacceptably high degree of intersample variation. If the effects of chemical or physical pretreatment on subsequent chemical absorption are to be studied, ethical considerations may preclude these studies being done in man. These limitations also apply to the recently developed living skin equivalent (LSE) models and *in vitro* animal studies.

Isolated perfused skin studies, such as the isolated perfused porcine skin flap (IPPSF) developed in our laboratory and described in this chapter, may be the "missing link" in the hierarchy of classic *in vitro* and *in vivo* models. The primary advantages of isolated perfused systems relate to:

1. The presence of a functional cutaneous vascular system.
2. The ease of continuously sampling venous perfusate.
3. The ability to conduct mass-balance and metabolism studies.
4. The availability of a large dosing surface area.
5. The capability of simultaneously assessing transdermal chemical flux and biomarkers of cutaneous toxicity.
6. The ability to conduct morphological evaluations at the end of a study in the same preparation that an absorption study was conducted.

7. The ease with which experimental conditions (temperature, humidity, perfusate flow and composition) can be manipulated and controlled without being concerned with interference from systemic feedback processes as is usually seen *in vivo*.

Isolated kidney, liver, and lung perfusions have been recognized as mainstay models for toxicology and pharmacology for many decades. Part of their acceptance relates to the ease of harvest since these organs are all characterized by having "closed" vascular systems with anatomically identifiable arterial inputs and venous outputs, both amenable to catheterizations with minimal expertise in surgery. In contrast, outside of the possible exception of ears, skin does not possess such a closed vascular system.

Rabbit and pig ears have been used as perfused skin systems to assess percutaneous absorption of topically applied compounds (Behrendt and Kampffmeyer, 1989; de Lange et al., 1992; Celesti et al., 1992). We feel that a fundamental problem with these systems is that the skin of the pinna is different (hair density, adnexial structures) than other body sites and has a much greater degree of blood perfusion (Monteiro-Riviere et al., 1990; Monteiro-Riviere, 1993). Additionally, the vasculature is specialized because of the unique thermoregulatory demands placed on this appendage. Auricular arteries perfuse a complex tissue bed consisting of skin, subcutaneous tissue, muscle, and cartilage. We believe that these additional factors outweigh the obvious economic benefits of obtaining ears from laboratory animals or abattoirs. Also, reports have appeared sporadically in the literature on perfused pieces of animal and human skin being used in various studies (Feldberg and Paton, 1951; Kjaersgaard, 1954; Hiernickel, 1985; Kietzmann et al., 1991); however, none have ever been optimized or validated for percutaneous absorption studies.

40.2 OVERVIEW OF METHOD

40.2.1 Surgical Preparation and Perfusion

The IPPSF is a single pedicle axial pattern tubed skin flap created from the abdominal skin of weanling pigs. This area was selected because it is perfused by direct cutaneous arteries (superficial epigastric artery) and drained by the associated paired venous commitantes. This allows a tube of skin to be created whose sole vascular supply may be cannulated and perfused *ex vivo*. The formation of a tubed flap allows the wound edges to be apposed and after a short healing period of 2 days, the preparation only drains via the venous system. This area of skin has also been used to create the *in situ* rat/human skin flap system (Kreuger et al., 1985) and recently an isolated perfused human nontubed skin flap model (Kreidstein et al., 1991). The IPPSF is fully described in the original publications describing its use (Riviere et al., 1986; Monteiro-Riviere et al., 1987; Riviere and Monteiro-Riviere, 1991). In addition, our group has developed an isolated perfused equine skin flap for use in assessing percutaneous absorption of chemicals across horse skin (Bristol et al., 1991) and a perfused human tumor bearing flap for use in anticancer drug targeting investigations (Vaden et al., 1993).

The IPPSF is created in a two-stage surgical procedure (Figure 40.1) (Bowman et al., 1991). Two flaps are created on each pig using the right and left caudal superficial epigastric

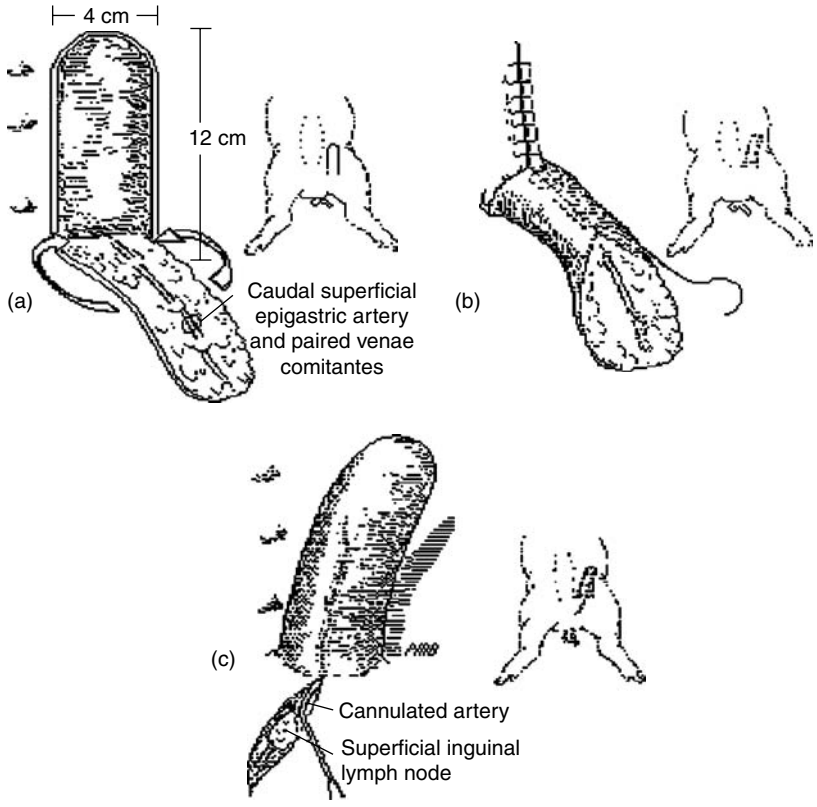


FIGURE 40.1 Two-stage surgical procedure used to create isolated perfused porcine skin flaps. A single pedicle axial pattern tubed skin flap is created in stage I (a and b) and harvested 2 days later in the stage II (c) procedure.

arteries. Depending on the experimental design, this allows one flap to serve as a control for the other during perfusion studies so as to minimize interflap variability. In stage I surgery, conducted aseptically and under inhalational anesthesia, a 4×12 cm area of skin previously shown to be perfused by this artery is demarcated, excised, tubed, and allowed to remain on the pig. Two days later, a time found to be optimal based on morphological criteria (Monteiro-Riviere et al., 1987), the flap is excised and the artery cannulated in a simpler stage II procedure. Both flaps are then removed and placed in the isolated perfusion chambers described later. The small incision remaining on the pig is allowed to heal and then the pig can be returned to its prior disposition (sale, other uses).

The isolated perfusion apparatus depicted in Figure 40.2 is a custom Plexiglas chamber designed to maintain the skin flap in a temperature- and humidity-regulated environment. Perfusion pressure, flow, pH, and temperature are set for desired conditions dictated by the experimental design and continuously monitored. The perfusion media is a modified Krebs–Ringer bicarbonate buffer (pH 7.4, 350 mOsm/kg) containing albumin (45 g/L) and supplied with glucose (80–120 mg/dL) as the primary energy source. Albumin is added to provide the oncotic pressure required to maintain capillary patency as dictated by Starling's laws, and to facilitate the absorption of lipophilic penetrants that otherwise would not be soluble in a pure aqueous buffer system. Normal perfusate flow through the skin is maintained at 1 mL/min/flap (3–7 mL/min/100 g) with a mean arterial cannula pressure ranging from 30 to 70 mmHG. With this system, flaps may be maintained biochemically and morphologically viable for up to 24 h. Two experimental configurations are possible for flap perfusion: recirculating and nonrecirculating. For most studies, the single pass nonrecirculating system is used. Our laboratory has perfused over 3200 IPPSFs and several hundred more have also been independently perfused in nonacademic laboratories to which the technique has been transferred.

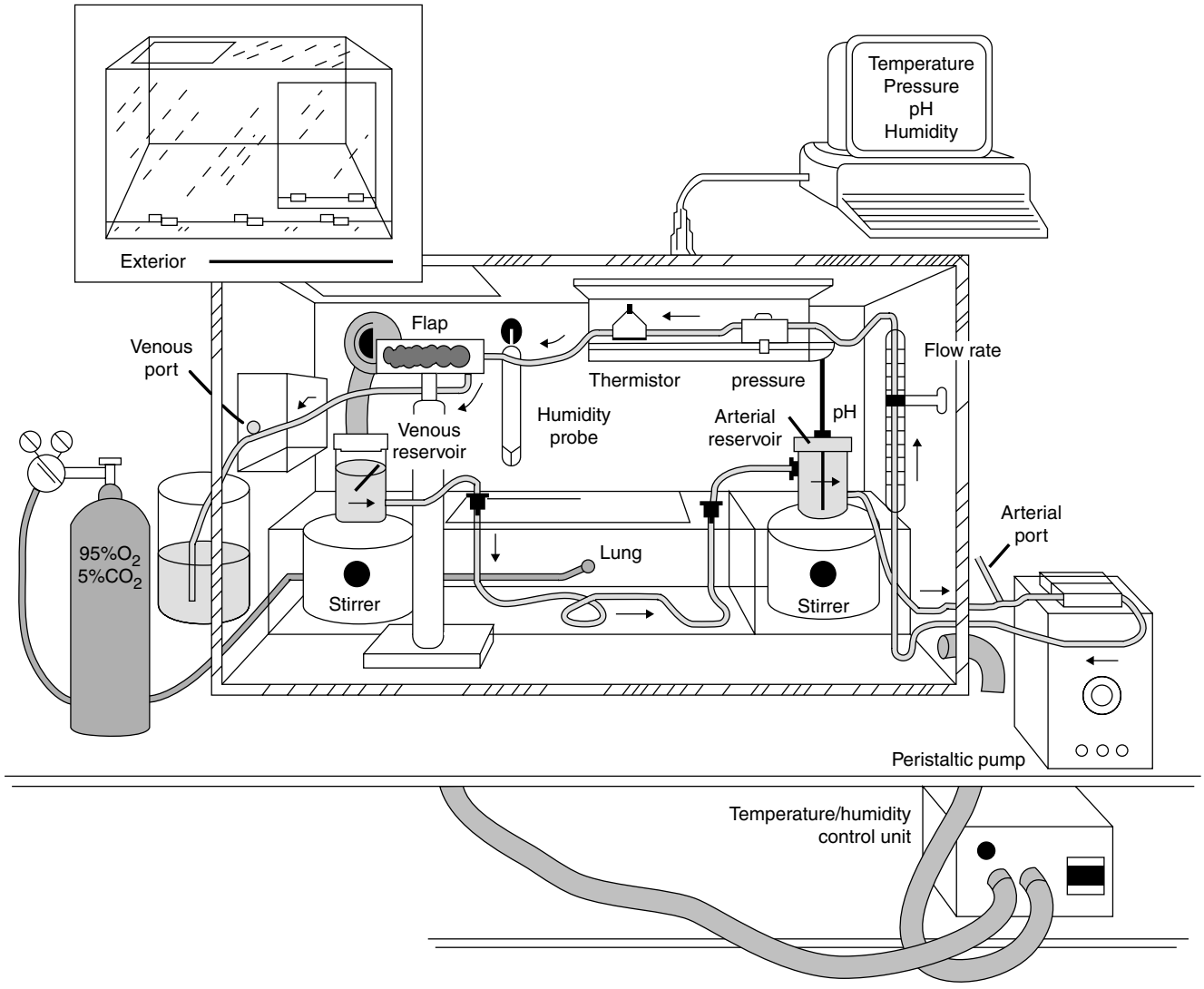


FIGURE 40.2 Temperature- and humidity-controlled chamber used to maintain IPPSF viability and environmental conditions throughout an experiment.

40.3 APPLICATIONS

There have been three general types of studies conducted in the IPPSF: toxicology, percutaneous absorption (including biotransformation and pharmacokinetic modeling), and cutaneous drug distribution (drug administered by intra-arterial infusion) studies. Intra-arterial drug distribution studies have been conducted to model the mechanism of drug distribution out of the capillary bed into the skin (Williams and Riviere, 1989a). The first two of these will be addressed in this chapter.

40.3.1 Assessment of Flap Viability and Development of Biomarkers for Toxicity Assessment

Viability of the preparations is monitored real time by assessing perfusate pressure and glucose utilization. We have found that during perfusion of a normal IPPSF, the most sensitive indicator of vascular function, and thus of vascular toxicity in dermatotoxicology experiments, is the parameter of vascular resistance calculated as perfusate pressure divided by flow. This parameter has also been used as an endpoint in pharmacological experiments when autonomic drug activity has been studied (Rogers and Riviere, 1994). Glucose utilization, calculated from the arterial-venous extraction ratio and perfusate flow, has been used as a marker of direct cutaneous toxicity of chemicals (King and Monteiro-Riviere, 1990; King et al., 1992; Monteiro-Riviere, 1992; Srikrishna et al., 1992) with decreases in cumulative glucose utilization being suggestive of direct chemical toxicity. However, glucose utilization may also be dependent on the extent of capillary perfusion, since only cells that are being perfused are capable of extracting glucose from the arterial perfusate (Rogers and Riviere, 1994). A decrease in glucose utilization is definitely a manifestation of chemical activity; however, a chemical-induced decrease in epidermal glucose utilization may be blunted by increased capillary perfusion.

Depending on the experimental design, a number of more specialized markers of viability, or loss thereof, may be assessed. Previously, we have assessed lactate production as a marker of epidermal glucose utilization and have observed decreased lactate production coexistent with decreased glucose utilization. Also, we have monitored the release of inflammatory mediators into the perfusate as biomarkers for physical- or chemical-induced toxicity. These have included PGE_2, $PGF_2\alpha$, and interleukins 1 and 8 as indicators of cutaneous inflammation (Monteiro-Riviere, 1992; Zhang et al., 1995a,b). Prostaglandin fluxes changed with compounds that altered vascular resistance. We have used these prostaglandin fluxes as endpoints in pharmacologic intervention studies designed to block a cutaneous toxicant's effect by preexposure infusion of a specific antagonist. For example, to dissect out the role of prostaglandins in sulfur mustard (HD)-induced cutaneous vesication, we demonstrated that perfusion with the nonsteroidal anti-inflammatory drug (NSAID) indomethacin blunted both PGE_2 release and altered vascular resistance, but did not completely prevent blister formation (Zhang et al., 1995b). Similarly, infusion of pyridostigmine bromide modulated interleukin 8 and PGE_2 release was seen after exposure to topical irritants (Monteiro-Riviere et al., 2002).

The final markers of dermatotoxicity are the myriad of morphological endpoints, which may be assessed easily at the termination of an experiment. Specimens are routinely collected for light microscopy to evaluate skin viability and integrity (Monteiro-Riviere et al., 1987). These studies are pivotal in assessing the nature of cutaneous toxicity produced (Monteiro-Riviere, 1992; Monteiro-Riviere et al., 2001). For a more specific insight into the mechanism of an observed effect, transmission electron microscopy may also be performed (Monteiro-Riviere et al., 1987; Monteiro-Riviere, 1990). These studies give a better indication of what is actually occurring within the epidermal cells at a level before light microscopy or gross observation indicate an adverse effect. For even more specific details, specialized morphological procedures may be conducted. These have included enzyme histochemistry to probe biochemical pathways affected by cutaneous toxicants (King et al., 1992; Srikrishna et al., 1992), immunohistochemistry, and immunoelectron microscopy to study the specific molecular targets involved in vesication secondary to chemical alkylation (King et al., 1994; Monteiro-Riviere and Inman, 1995; Zhang et al., 1995c) or topical jet fuel exposure (Rhyne et al., 2002), and x-ray diffraction microscopy to probe path ways of metal penetration (Monteiro-Riviere et al., 1994a).

Although every one of the aforementioned biomarkers may be assessed in other skin models, the unique strength of the IPPSF is that all may be simultaneously evaluated in the same preparation. For example, we have demonstrated the utility of the IPPSF to serve as a humane *in vitro* model for UVB phototoxicity (Monteiro-Riviere et al., 1994b). In these studies, physiological parameters such as vascular resistance, glucose utilization, and prostaglandin (PGE_2) efflux could be simultaneously evaluated in the same preparation as morphometric quantitation of pyknotic "sunburn" cells and estimation of epidermal growth fraction using histochemical staining for the proliferating cell nuclear antigen (PCNA). In chemical-induced dermatotoxicity, compound flux through the skin can simultaneously be determined in the same preparation that physiological and morphological endpoints are being evaluated. Such studies have been conducted with paraquat (Srikrishna et al., 1992), lewisite (King et al., 1992), 2-chloroethyl methyl sulfide (King and Monteiro-Riviere, 1990), lidocaine iontophoresis (Monteiro-Riviere, 1990), and electroporation (Riviere et al., 1995).

This approach offers many unique advantages. First, it guarantees that cutaneous exposure to a penetrating molecule actually occurred. Second, it allows quantitation of this exposure and subsequent correlation to severity of toxicity observed. Finally, it would allow the development of linked toxicokinetic–toxicodynamic models to be developed, which should shed insight into the mechanisms of cutaneous toxicity.

40.3.2 Absorption Studies

The IPPSF has also been extensively utilized to quantitate the cutaneous penetration and absorption of topically applied

compounds for which systemic exposure could result in toxicity. There are a number of levels of sophistication which can be employed depending on the nature of the penetrant and the precision desired. The simplest approach is to measure compound flux into the venous perfusate and express this as the percent of applied dose absorbed (Figure 40.3). The compound remaining on the skin surface and within the skin can be easily assessed, especially if radiolabelled chemical was employed. This method is accurate if most of the absorption is complete at the end of an experiment (e.g., venous fluxes are approaching background). The amount of penetrated chemical in the venous effluent may then be determined as the area under the curve (AUC) of the venous efflux profile.

Numerous IPPSF absorption studies have been conducted and are referenced in manuscripts included in the reference section of this chapter. Recent IPPSF absorption studies have been conducted on 2,6-Di-*tert*-butyl-4-nitrophenol (DBNP) (Inman et al., 2003), nonylphenol and nonylphenol ethoxylates (Monteiro-Riviere et al., 2003), the jet fuel JP-8(+100) (Muhammad et al., 2004) as well as Gulf Way syndrome related chemicals discussed later. A great deal of very recent work has been done with complex chemical mixtures (Riviere and Brooks, 2007), a topic covered in Chapter 6 of this test, which presents details on some of these IPPSF studies.

At the end of a topical treatment, additional studies may be conducted to determine the amount and distribution of penetrated compound within local tissues. The most precise technique available for this purpose is to take a core biopsy through the dosing site, snap freeze it in liquid nitrogen, and then cut serial sections to precisely localize chemical distribution within the skin as a function of penetration depth. In these studies, which are fully described in the literature (Riviere et al., 1992a; Monteiro-Riviere et al., 1993), the surface of the application site is first gently washed with a mild soap solution and then dried with gauze. Cellophane tape is then applied to "strip" is stratum corneum. A biopsy punch is used to take the core of tissue, which is then embedded in OCT compound, quenched in an isopentane well cooled by liquid nitrogen and immediately stored at −80ºC until it is sectioned in a cryostat. Each tissue section (representing a disc of skin containing radiolabelled compound), along with the washes and tape strips, is then combusted and radioactivity determined using liquid scintillation spectroscopy. The resulting data are a depth penetration profile for the compound under study (Figure 40.4). Although similar studies may be conducted *in vivo*, the advantage of the IPPSF is that this data are obtained in the same preparation that venous flux of the compound is determined, allowing the investigator to assess factors that modulate tissue penetration separate from absorption into the vasculature. Figures 40.3 and 40.4 show the different pattern seen from these two perspectives. This approach was also utilized to assess the effect of various environmental exposure variables on the absorption of TCB (Qiao and Riviere, 2000). Finally, a technique was recently

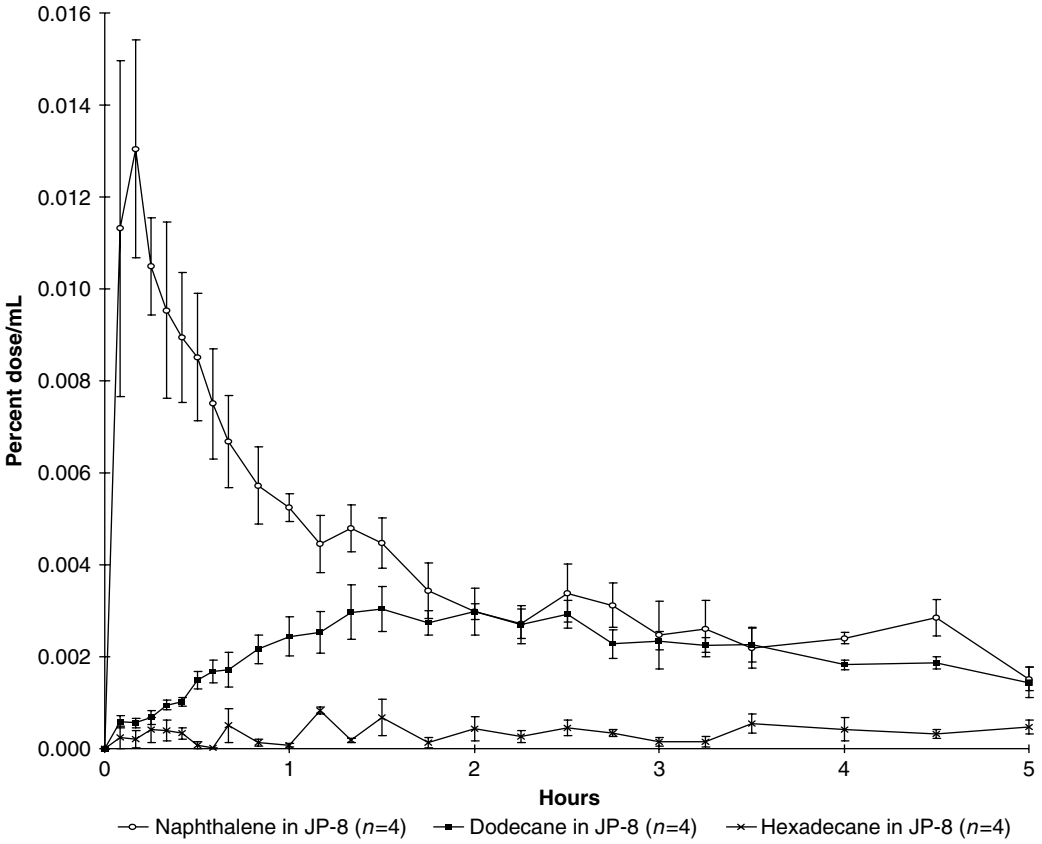


FIGURE 40.3 IPPSF venous flux profiles (naphthalene, dodecane, hexadecane) for a percutaneous absorption experiment (mean ± SD).

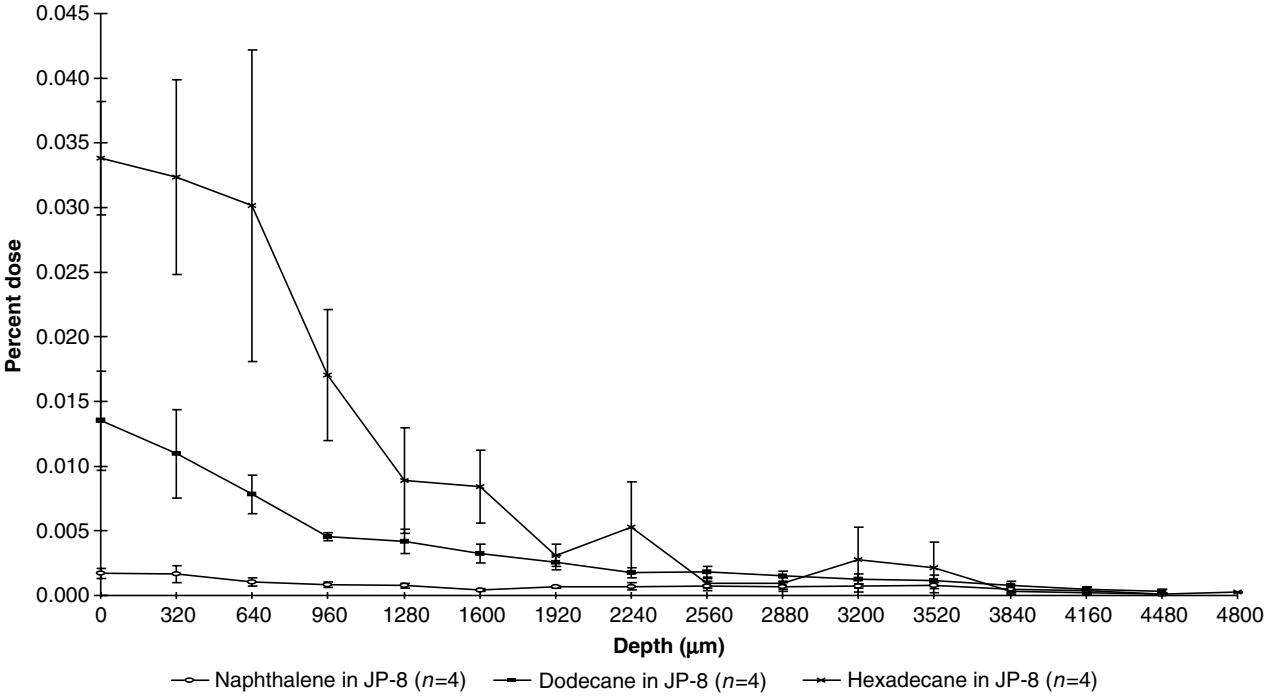


FIGURE 40.4 IPPSF tissue distribution profile at the end of an experiment for the same compounds as in Figure 40.3 (naphthalene, dodecane, hexadecane), seen after topical administration (mean ± SD).

developed to assess the absorption of volatile compounds in this IPPSF (Riviere et al., 2000).

Percutaneous absorption in the IPPSF was correlated ($r^2 \approx 0.8$) to *in vivo* human absorption for five diverse compounds (Wester et al., 1998). The IPPSF estimate for absorption used was the amount absorbed into the perfusate plus the amounts penetrated into the skin. Comparative absorption values (% Dose; Mean ± SD) were:

Compound	Human	IPPSF
Salicylic acid	6.5 ± 5	7.5 ± 2.6
Theophylline	16.9 ± 11.3	11.8 ± 3.8
2,4-Dimethylamine	1.1 ± 0.3	3.8 ± 0.6
Diethyl hexyl phthalic acid	1.8 ± 0.5	3.9 ± 2.4
ρ-Aminobenzoic acid	11.5 ± 6.3	5.9 ± 3.7

Figure 40.5 illustrates the IPPSF to *in vivo* human correlation for all compounds studied to date for which both sets of data exist.

40.3.3 Dermatopharmacokinetic Studies

The greatest level of precision that may be achieved with this system is to apply pharmacokinetic models to either extrapolate to the *in vivo* situation or quantitate the fate of drug within the skin. These are especially adaptable to a system such as the IPPSF because venous drug efflux can be readily determined, which is the starting point for the analysis. These strategies are outlined in Figure 40.6. If the goal of the study is to predict *in vivo* disposition, then one should view the IPPSF as a "living" infusion pump whose output

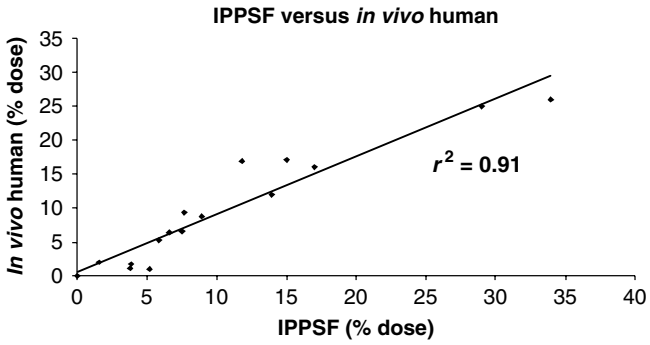


FIGURE 40.5 IPPSF predicted versus *in vivo* human dermal absorption for 16 compounds for which data sets are available.

flux (venous efflux) is actually the input into the systemic circulation. First, this approach allows one to use porcine skin data to model human skin penetration with human systemic pharmacokinetic data to avoid interspecies differences in drug distribution, metabolism, or elimination. Second, this strategy allows one to predict the actual serum drug concentration-time profile that may be seen *in vivo*. This approach has been used to predict the *in vivo* disposition of a number of drugs, including arbutamine and LHRH (Riviere et al., 1992b; Heit et al., 1993; Williams and Riviere, 1994). The systemic input may either be the observed IPPSF venous efflux profile or the pharmacokinetic simulation of this profile.

This brings us to the second use of pharmacokinetic modeling, which is to predict the shape of the cutaneous efflux profile based on factors governing the absorption and distribution of the drug. Our group initiated these studies using drug infused into the arterial cannula whereby arterial and venous

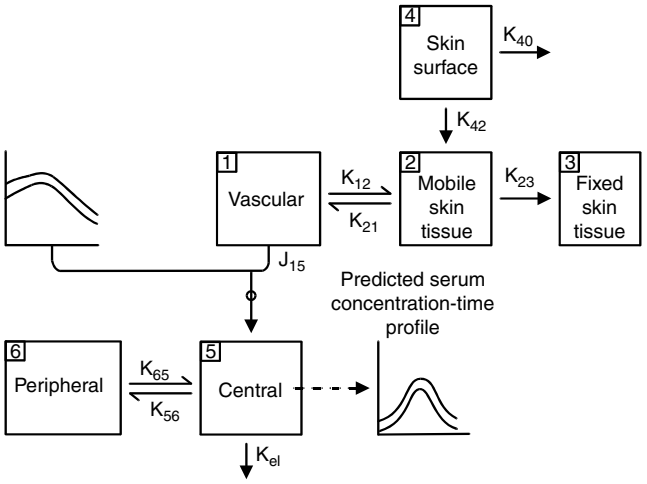


FIGURE 40.6 Conceptual approach to using IPPSF absorption profile (upper left) or dermatopharmacokinetic model (upper right) as input into a systemic pharmacokinetic model (lower left) to predict an *in vivo* serum concentration-time profile.

extraction of the drug could be determined. This approach allowed the basic structure of our IPPSF model to be determined (Williams and Riviere, 1989a). The specific volumes of the extracellular and intracellular spaces were then validated using dual radiolabelled albumin and inulin infusions (Williams and Riviere, 1989b). The next step was to add a percutaneous absorption component (Williams et al., 1990; Carver et al., 1989), which is the basic model depicted in Figure 40.6. This approach allows one to conduct an experiment over an 8 h period and use the venous efflux profile to determine the parameters of the pharmacokinetic model. If the venous efflux profiles demonstrated a peak or beginning of a plateau phase, then using the model parameters, the 8 h data may be extrapolated to extended time points. Such correlations ($r^2 \approx 0.9$) were determined between extrapolated IPPSF profiles and observed 6 day absorptions for a number of diverse compounds, further demonstrating both the utility of the IPPSF to predict percutaneous absorption in humans, as well as the underlying similarity between pig and human skin (Riviere and Monteiro-Riviere, 1991). Since only the total fraction of a topically applied dose absorbed was predicted in these situations, *in vivo* pharmacokinetic data were not needed because a blood concentration-time profile was not available.

The only data used in the models presented earlier are actual venous efflux profiles and residual compound recovered at the end of an experiment (e.g., unabsorbed chemical bound to dosing device, skin surface wipes, and drug in the flap). The precision of such models may be greatly improved if the tissue penetration data described (stratum corneum residues by tape strips, serial sections of biopsy cores) are also included in the data analysis. Additionally, if other *in vitro* data such as stratum corneum partition coefficients and rates of evaporation are independently determined in porcine skin (Williams et al., 1994), more sophisticated models may be developed, which can shed much greater insight into the mechanisms governing chemical absorption and penetration. Figure 40.7 depicts such a model

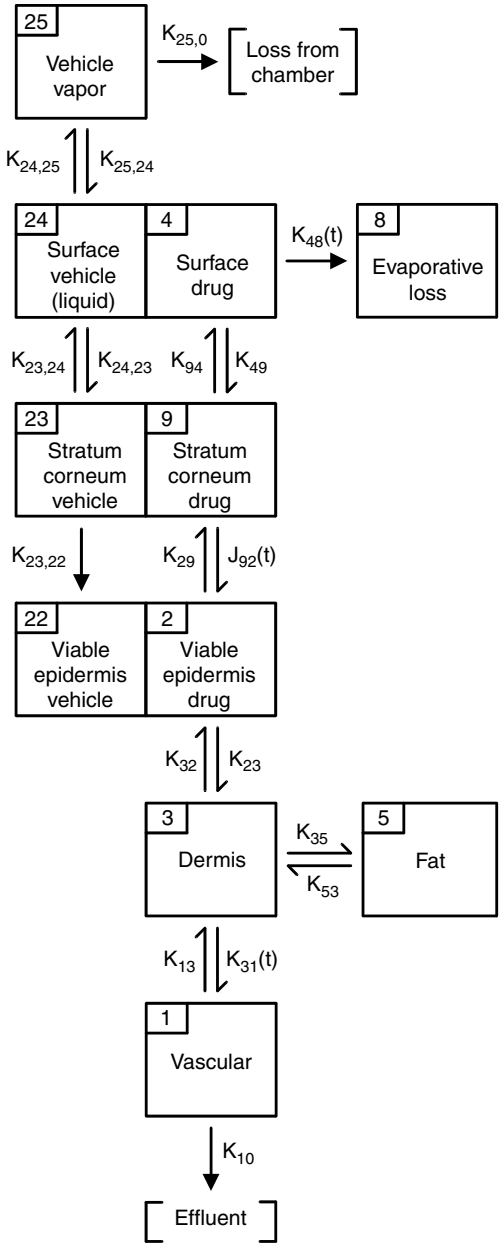


FIGURE 40.7 Complete dermatopharmacokinetic model of a penetrant and its vehicle (shaded), utilizing multiple data points obtained from IPPSF experiments (venous effluent profiles and tissue samples as seen in Figures 40.3 and 40.4) and parameters from parallel *in vitro* studies.

which incorporates the fate of the vehicle used to apply the drug, since it is widely acknowledged (but seldomly modeled) that vehicle affects the rate and extent of penetration of many compounds (Williams and Riviere, 1995). This approach allows one to take into account penetrating chemical or vehicle interactions with stratum corneum lipids (e.g., enhancers such as Azone®), which could alter permeability, to be directly incorporated into the analysis. The work has been extended to study the simultaneous absorption of multiple (>2) penetrants so that a mechanistic approach to assessing exposure to chemical mixtures may be developed (Riviere et al., 1995). This approach is now being applied to the complex

absorption patterns seen in topical jet fuel exposure (Riviere et al., 1999). Finally, the absorption of toxic compounds which may alter their own absorption, secondary to cutaneous toxicity of the penetrant, has been studied using the chemical vesicant sulfur mustard (Riviere et al., 1995). In this model, absorption profiles could only be precisely described if the vascular compartment was modulated as a function of sulfur mustard in the skin. This was independently correlated to vascular volume/permeability using inulin infusions to measure vascular space.

The major limitation to all pharmacokinetic approaches such as these relate to the large data requirements needed to solve model parameters. A full solution for a model, such as presented in Figure 40.7, requires a series of replicated experiments using a single chemical applied at different doses and experiments terminated at various time points. As mentioned earlier, *in vitro* studies would be conducted to obtain specific biophysical parameter estimates. All data are simultaneously analyzed. For many compounds, specific components of the full model may not be required and thus in reality, the actual model fitted is simpler. Statistical algorithms are presently being developed to select the optimum model for the specific compound being studied and collapse the remainder of the model structure into a matrix from which individual rate parameters cannot be extracted (Smith et al., 1995). This work has now resulted in the collapse of an equation that describes an IPPSF efflux profile to a three parameter equation: $Y(t) = A\ (e^{-bt} - e^{-dt})$, which adequately describes most IPPSF flux profiles (Riviere et al., 2001). This approach allows emphasis to be placed only on those compartments or processes that are important for the chemical being studied, yet retains the general structure of the model for all compounds so that future extrapolations are facilitated.

40.3.4 Cutaneous Biotransformation

The final aspect of assessing percutaneous absorption, which has not been considered up to this point, is cutaneous biotransformation. The IPPSF is ideally suited for this purpose and has been used to study metabolism of pesticides, drugs, and endogenous compounds (Bikle et al., 1994; Carver et al., 1990; Chang et al., 1994; Riviere et al., 1996). Specific pharmacokinetic models which incorporate IPPSF data and *in vivo* disposition have been constructed (Qiao et al., 1994; Qiao and Riviere, 1995). These studies demonstrate a number of important features of percutaneous absorption of chemicals which are biotransformed during passage through the skin. The method of dose application significantly affects the metabolic profile observed in the venous efflux. Occlusion enhances the fraction of parathion metabolized to para-nitrophenol both in the IPPSF and the *in vivo* pig. The mechanism of this effect has not been determined, although it illustrates the inherent complexity of skin relative to assessing the fate of chemicals applied on its surface. By constructing dermatopharmacokinetic models to address these phenomena, quantitative parameters describing absorption and cutaneous distribution independent of biotransformation may be used as experimental endpoints.

The primary implication of biotransformation to risk assessment is that the *in vitro* to *in vivo* extrapolation strategy outlined in Figure 40.6 is actually oversimplified since it assumes that all inputs from the skin to the general circulation are in the form of parent chemical. In reality, multiple inputs from the skin to systemic circulation are required in making the extrapolation process more complex. *In vivo* work requires that studies be done both intravenously and topically so that systemic and cutaneous metabolism may be separated. Dosing methods must also be assessed if effects such as occlusion are to be quantitated.

40.3.5 Percutaneous Absorption of Vasoactive Chemicals

One of the major advantages of using an isolated perfused tissue preparation is the presence of an intact vascular system with dermal microcirculation. This is important from the perspective of assessing the effect of altered blood flow on compound disposition, as well as determining how a penetrating chemical's inherent vasoactivity affects its own fate. Unlike other organ systems, the range of blood flow possible through mammalian skin is tremendous because of its role in thermoregulation. The primary impact of altered dermal perfusion on the disposition of penetrated chemical may be on the surface area of the exchanging capillaries being perfused, which determines the actual volume of dermis that is perfused and is thus available for systemic absorption (Riviere and Williams, 1992; Williams and Riviere, 1995). Alternatively, changes in dermal perfusion resulting from modulation of arterial-venous shunt activity may completely bypass areas of skin or result in deeper dermal penetration, a phenomenon observed *in vivo* with piroxicam (Monteiro-Riviere et al., 1993). Changes in dermal perfusion may be initiated by physiological homeostatic mechanisms, by exposure to vasoactive drugs or secondarily by chemical-induced irritation with concomitant release of vasoactive inflammatory mediators (e.g., prostaglandins). Using glucose utilization as a measurement of exchanging capillary perfusion, we have recently begun to map out the IPPSF vascular response to the infusion of vasoactive drugs in an attempt to experimentally define the pharmacodynamics of vasoactive drugs in this system for future integration into a comprehensive pharmacokinetic–pharmacodynamic model (Rogers and Riviere, 1994).

The impact of a drug's vasoactivity on its rate and extent of percutaneous absorption and distribution within skin can best be illustrated with IPPSF studies on the iontophoretic transdermal delivery of lidocaine coadministered with the vasodilator tolazoline or the vasoconstrictor norepinephrine (Riviere et al., 1991, 1992a). Coiontophoresis of both these compounds using *in vitro* diffusion cell systems resulted in essentially no effect on lidocaine flux. However, identical *in vivo* dosing conditions resulted in increased blood concentrations when tolazoline was present. As can be seen in Figure 40.8, tolazoline enhanced and norepinephrine decreased lidocaine flux in IPPSF studies. When one examined the concentrations in the skin underlying these electrodes, the opposite

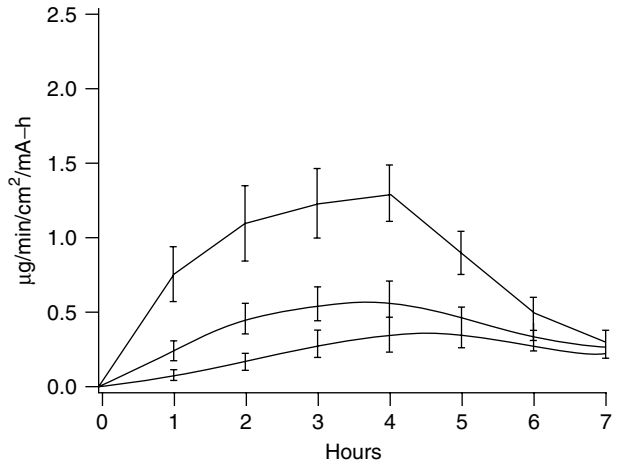


FIGURE 40.8 IPPSF venous efflux profile of iontophoretically delivered lidocaine (—) demonstrating vascular effect with enhanced delivery after tolazoline (——) and reduced delivery after norepinephrine (———) coadministration.

pattern was seen. These vascular effects have now been incorporated into our dermatopharmacokinetic model (Williams and Riviere, 1993). These studies clearly demonstrate the importance of the microcirculation on determining the nonsteady state profile of drug delivery and dermal disposition.

40.3.6 Effect of Mixtures and Vascular Drug Infusions on Dermal Absorption

One recent series of studies nicely illustrates the strength of a perfused skin model such as the IPPSF. These investigations focused on the mutual interactions among DEET, permethrin, and pyridostigmine bromide, as well as other military exposure scenarios (pesticides, CW agents) on DEET and permethrin dermal absorption relative to their potential role in the Gulf War syndrome (Baynes et al., 2002; Monteiro-Riviere et al., 2003; Riviere et al., 2002, 2003; Baynes, 2006). These studies clearly demonstrated interactions between topically applied DEET and permethrin on each other's dermal absorption. However, the most interesting finding was that systemic infusion with pyridostigmine bromide, in contrast to the other agents studied, significantly increased permethrin dermal absorption almost eightfold, and modified cytokine release seen after topical mixture exposure. Few percutaneous absorption studies have studied the effect of systemic exposure on subsequent dermal absorption. These studies also documented the effect of these complex exposure scenarios on drug masses in the epidermis and dermis, in some cases resulting in patterns different from what was transported into the perfusate, similar to what was previously depicted in Figures 40.3 and 40.4 for jet fuel aliphatic hydrocarbons.

40.4 DISCUSSION

The earlier presentation provides the reader with an overview of the uses of a perfused skin model such as the IPPSF in percutaneous absorption and dermatotoxicokinetic studies. One of its major advantages is that both absorption and toxicity may be assessed in the same preparation. The pharmacokinetic models developed are experimentally verifiable. The major limitations are centered on the cost of the preparation and the technical expertise required to successfully conduct the studies. The overall cost is significantly greater than *in vitro* diffusion cell studies or *in vivo* rodent experiments, comparable to human skin equivalent and larger mammal (dog, pig, primate) *in vivo* work, and is much less expensive than human trials. However, cost alone is not a sufficient criterion. These studies are humane; and more information may be gathered than is obtainable with either *in vitro* or *in vivo* work. Optimal benefit may be achieved if these studies serve as a bridge between *in vitro* human/animal and *in vivo* animal work and the ultimate *in vivo* human exposure scenario.

40.4.1 Integrated Approach to Dermal Risk Assessment Using a Dermatopharmacokinetic Template

The optimal method to assess all of the aforementioned complex events is to use a hierarchy of experimental model systems ranging from *in vitro* diffusion cells for animals and humans to perfused skin studies such as the IPPSF to *in vivo* animal and human studies. By designing such experiments using a comprehensive dermatopharmacokinetic model as a template, the limitations of each system may be delineated and a complete understanding of the rate-limiting steps in the process defined. For example, there is general consensus that the major biological differences between humans and other species in regard to a chemical's percutaneous absorption is the nature of the stratum corneum lipids and patterns of biotransformation. As others have documented, the lipids of the stratum corneum of the pig are very similar to man and may be the primary reason that with *in vivo* comparisons, pigs and humans are often very similar. Such data are often not available relative to biotransformation. However, patterns of biotransformation may be determined from *in vitro* human diffusion cell or skin-equivalent studies and can be directly compared to *in vitro* pig data collected under identical conditions. Any differences observed may then be incorporated into the dermatopharmacokinetic model. The limitations to solely rely on the *in vitro* human data relate to the lack of proper anatomical orientation and microcirculation, which could alter the rate of parent chemical and metabolite penetration and thus pattern of biotransformation.

Any prediction errors purely inherent to the *in vitro* to *in vivo* extrapolation may be studied directly in the pig and a correction vector incorporated into the kinetic template. With these limitations defined, reasonable extrapolations to humans may then be made. Importantly, physiological or pathological processes which have been theoretically or experimentally shown to be important in either *in vitro* human or *in vivo* animal studies may be simulated. Using this approach, these hypotheses may then be tested in a reduced number of human clinical studies. More importantly, for many extremely toxic chemicals, human studies are never possible due to ethical

limitations. This makes the pharmacokinetic approach, capable of synthesizing and integrating data from many levels of experimentation, an optimal strategy for human risk assessment. The same holds for drugs in very early stages of preclinical development.

In conclusion, the IPPSF appears to be a useful humane experimental model system for assessing both a chemical's percutaneous absorption profile and dermatotoxic potential. The IPPSF's greatest strength is the ability to experimentally characterize both phenomena simultaneously. By utilizing a fixed template to guide experimental design, one is assured that maximum information may be obtained from each individual study while maintaining the ability to extrapolate across chemicals.

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41 Physiologically Based Pharmacokinetic Modeling

James N. McDougal

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41.1 INTRODUCTION

Understanding and quantifying the penetration of chemicals into and through the skin is important in both pharmacology and toxicology. In nearly every case, the species of interest is the human species, although laboratory animals are often used as surrogates, particularly in the case of toxicological studies. Appropriate use of laboratory animals necessitates understanding differences between species so that the process of extrapolation to humans is meaningful. This is vital for *in vivo* animal studies, which are often more complex than *in vitro* animal studies. *In vivo* studies have the advantage of intact skin that has blood flow, is alive, and is responsive. Metabolism, nervous, and humoral responses are also present and, therefore, living skin more accurately reflects human exposure scenarios. Traditionally, the analysis of *in vivo* skin penetration in laboratory animals has involved estimation of the amount of chemical that has penetrated using either blood concentrations or the amount of chemical excreted after a dermal exposure. These methods are descriptive; applicability of the results is limited by the appropriateness of the specific experimental design and the similarities between the laboratory species chosen and humans.

Owing to the increase in the availability of computer hardware and software over the last three decades, methods that are based on physiological and pharmacokinetic principles are now feasible alternatives for analysis of *in vivo* skin penetration. These physiologically based pharmacokinetic (PB-PK) approaches mathematically describe the dynamics of chemicals in the body in terms of rates of blood flow, permeability of membranes, and partitioning of chemicals into tissues. Characterizing absorption in terms of parameters, which are measurable and species specific, facilitates extrapolations to the real species of interest, providing these parameters are known or can be determined for humans. This chapter describes PB-PK models, their use as a tool to quantify and understand the process of dermal absorption and penetration, and their suitability for dose, route, and species extrapolation.

41.2 WHY USE PB-PK MODELS?

One of the big advantages of dermal PB-PK models over traditional *in vivo* methods is the ability to accurately describe nonlinear biochemical and physical processes. Describing skin penetration based on blood concentrations or excretion rates, as "percent absorbed," assumes that all processes have a simple linear relationship with the exposure concentration. When nonlinear processes occur in the absorption, distribution, metabolism, or elimination of a chemical, describing penetration as "percent absorbed" does not provide information that can be applied to situations other than the experimental situation. Skin penetration may not be linear when there is binding or metabolism in the skin or when skin blood flow is a limiting factor. Many biochemical processes in the body are nonlinear, for example the percent of chemical metabolized per hour at a low liver concentration may be much greater than the percent metabolized per hour at a high liver concentration. A quantitative description of saturable kinetics in the model may allow it to be predictive of blood or tissue concentrations from various doses. A complete mathematical description of dermal pharmacokinetics takes mass balance throughout the animal into account, and makes it possible to estimate fluxes (amount/time) and permeability constants (distance/time). These expressions of the penetration process are required to accurately predict penetration in other situations (that is, different exposure area, time, or concentration) when nonlinear processes are present.

A properly validated PB-PK description of the skin will provide more information from each experiment than is possible without it. For example, if it is the chemical concentration in an organ or tissue that is important, by understanding the quantitative relationship between blood concentrations and tissue concentrations, serial blood sampling may provide the estimate of the tissue dose that is required without the need for an invasive procedure to sample tissue concentrations. Another good example would be the estimation of rate of metabolism in the skin. Proper comparison of a PB-PK description of metabolite production after an intravenous infusion with the rates of metabolite production after application to the skin at several concentrations allows the metabolic parameters in the skin to be estimated.

In this age of increased concern over the use of animals in research, it is important to try to reduce animal use and get maximum information from each animal that must be used. Before any experimentation, PB-PK models can often be used to form predictions that will help in designing experimental doses and sampling times, thus avoiding "range finding" experiments. During the experiment, PB-PK descriptions may allow the use of fewer animals because it may not be necessary to sacrifice animals at various time points to get tissue concentrations. After the study is complete, PB-PK models allow one to extrapolate results to other exposure areas, times, or concentrations, possibly eliminating the need to repeat an experiment under different conditions.

Another important reason for using PB-PK modeling of skin penetration is to acquire the experience necessary to extrapolate to other species. Classical pharmacokinetic modeling assumes that the body can be adequately described by one to three compartments based on the shape of the semilogarithmic plot of plasma concentration versus time (Gibaldi and Perrier, 1982). The most common classical description is a two-compartment linear system where one compartment is the plasma and the other all the remaining body water and tissues. Using this type of model, the plasma concentration curve can be fit by a distributive phase and a postdistributive phase. This type of model is useful in clinical situations for determining dose or dose regimen. Classical modeling has occasionally been used in skin penetration studies (Cooper, 1976; Wallace and Barnett, 1978; Peck et al., 1981; Chandrasekaran et al., 1978; Birmingham et al., 1979; Guy et al., 1982; Kubota and Ishizaki, 1986).

Figure 41.1 is a schematic representation of the classical two-compartment pharmacokinetic model having a body compartment connected with the plasma. The first-order transfer rates (K_{12}, K_{21}, K_{10}) are descriptive of a particular situation (Gibaldi and Perrier, 1982) but do not allow extrapolation to other exposure conditions or species because their physiological basis is obscure. PB-PK models are better suited for extrapolation because their physiological basis is well defined. It has been shown that a PB-PK model for the inhalation of styrene in rats can be predictive of blood and exhaled air concentrations of styrene in humans after scaling-up the physiological and metabolic constants (Ramsey and Andersen, 1984). Extrapolation with a PB-PK model is only limited

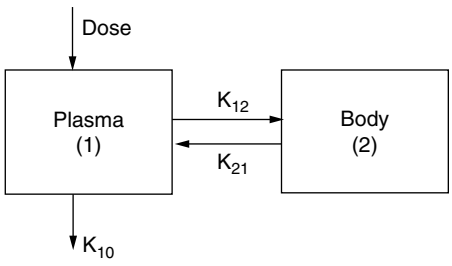


FIGURE 41.1 Classical pharmacokinetic model with two compartments and first-order transfer and elimination rates.

by the ability of the modeler to quantitatively describe the species differences in the pharmacokinetic and physiological processes involved.

41.3 WHEN CAN PB-PK MODELS BE USED?

PB-PK models can be used in nearly any *in vivo* experimental situation in which the physiological and pharmacokinetic processes can be described adequately for the purposes of the scientific question to be answered. It is often not necessary to have an exhaustive description of the animal to be studied—only the simplest description that "works." It is possible to imagine a PB-PK model that describes blood flow, partition coefficients, and metabolic characteristics for each organ in a specific mammal, but a single scientific question that would require such an exhaustive description could not be imagined! Normally it is sufficient to combine many organs into several lumped compartments that have similar blood flows and partition coefficients. The requirement for quantitative understanding of these conceptual processes is both the strong point and the Achilles heel of PB-PK modeling. Quantitative descriptions are the strong point because of their basis in underlying principles, but they are the weak point because the level of understanding required is not easy to achieve. Often the initial description of a particular process is not adequate but through experimentation and more careful description, based on sound pharmacokinetic and physiological principles, the fundamental understanding of the processes involved can be increased.

41.4 WHAT ARE THE COMPONENTS OF A PB-PK MODEL?

Simply speaking, a mammalian organism is comprised of diverse, sometimes metabolically active, pools of fluid separated by membranes which prohibit, permit, or promote passage of the fluids and their dissolved contents. These fluids and membranes obey and can, therefore, be described by the physical laws of fluid dynamics, transport, and diffusion. Skin is one of the most important membranes because it separates and protects animals from their environment. The major fluids, which contribute 60% of body weight, are blood plasma, interstitial fluids, and intracellular fluids. Plasma, the most important fluid because of its continuous motion, transports the red cells, white cells, platelets, and soluble components in the blood. Interstitial fluid, which bathes cells with three times the volume of the plasma, is diffuse and separated from the plasma only by capillary walls. The comparatively static intracellular fluid is separated from the extracellular fluids by specialized cell membranes with sophisticated transport systems. The membranes in the tissues that keep these fluids organized are protein–lipid structures of varying thicknesses, which may contain alterable apertures and carry metabolic enzymes. With this uncomplicated description as a basis, most pharmacokinetic processes can be simplified and described in terms of flows, volumes, solubilities, diffusion, and metabolic rates. When these physiological and biochemical processes can be quantified, a mathematical description can be constructed and compared with experiments to accurately describe the processes involved (see reviews by Himmelstein and Lutz, 1979; Lutz et al., 1980; Gerlowski and Jain, 1983; Clewell and Andersen, 1989).

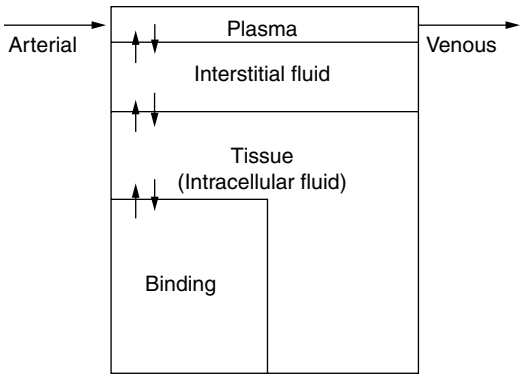


FIGURE 41.2 Diagrammatic description of a lumped compartment with three subcompartments and binding in the tissue subcompartment.

41.4.1 Tissue Compartments

The building block of a PB-PK model is the compartment. A compartment is a collection of fluids or tissues or organs that are grouped together because of similar physiological and pharmacokinetic characteristics rather than anatomical considerations (Lutz et al., 1980). Each lumped compartment receives inward flux of chemical in the blood flow, has a volume, and may incorporate binding or loss of chemical through outward flux or metabolism. Subcompartments may be necessary to accurately describe barriers to movement or sequestration of chemical. Figure 41.2 illustrates a lumped compartment.

Even this level of complexity is not always necessary to adequately describe the processes that are occurring. The transport of chemical across the thin capillary wall may be so rapid that the plasma and interstitial fluid have equivalent concentrations and, therefore, it may be possible to combine the plasma and interstitial fluid subcompartments into one extracellular fluid subcompartment. Diffusion across cellular membranes into the intracellular fluid may be so rapid that flow of the blood to the compartment is the rate-limiting factor affecting uptake of a chemical and, therefore, it may be possible to avoid subcompartments completely. The free concentration of chemical in the plasma, interstitial fluid, or intracellular fluid subcompartments will depend on whether binding or metabolism occurs in the subcompartment.

41.4.2 General PB-PK Model

Penetration of the skin is a process that lends itself to PB-PK modeling. Compartments are chosen based on an understanding of the pharmacokinetics of the chemical and the purpose for the model. Figure 41.3 shows a model with five simple compartments that was designed for predicting blood concentrations from different exposure times and concentrations

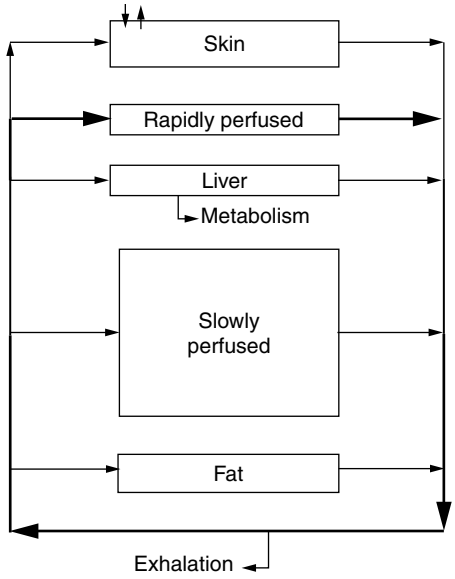


FIGURE 41.3 Diagrammatic representation of a PB-PK model with five simple compartments connected by blood flow. Compartment volumes and blood flows are approximately to scale.

on the skin. Each compartment is assumed to be well stirred, flow limited, and have no subcompartments. Potential losses of chemical are by evaporation from the skin, hepatic metabolism, and exhalation. The description is of the venous equilibration type, without blood volume being specified.

The skin compartment is discussed in detail in the next subsection. The rapidly perfused compartment lumps tissues with high blood flow and high affinity for the chemical. It represents kidney, viscera, brain, and other richly perfused organs. The slowly perfused compartment has low blood flow, low affinity for the chemical, and represents muscle and other poorly perfused tissues and organs. The fat compartment has low blood flow, high affinity for the chemical, and represents various types of fat. These characteristics are important criteria in choosing the compartments. According to this description, the sole route of entry for the chemical is the skin and elimination is by way of diffusion out of the skin followed by metabolism in the liver, and exhalation if the chemical is volatile. Additional compartments would be required for a chemical that is eliminated in the kidney, or if concentration in a target organ (e.g., testis) is of particular interest.

41.4.3 Skin Compartment

A skin compartment is just a special subset of tissue compartments that, because it is the defined portal of entry and has definable anatomy and physiology, needs to be further elaborated. Figure 41.4 illustrates a skin compartment that contains most of the anatomical detail that may be important in skin penetration (Bookout et al., 1996, 1997). Most of this detail will not be necessary for any particular chemical, but is described here for completeness. Each subcompartment communicates in both directions with adjacent compartments

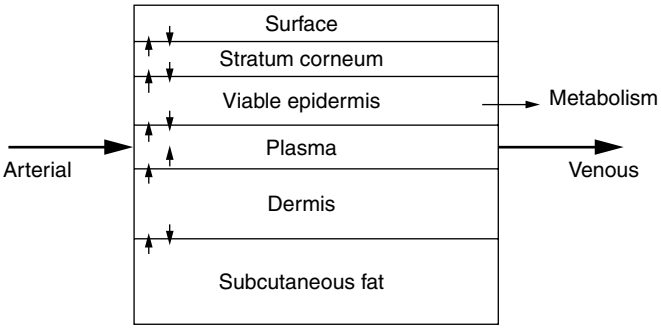


FIGURE 41.4 Diagrammatic representation of a skin compartment with six subcompartments and metabolism occurring in the viable epidermis.

and each has a concentration, volume, and affinity for the chemical of interest. The surface subcompartment, although not strictly part of the skin, is crucial to making the PB-PK model functional. The surface area exposed, exposure concentration, amount applied to skin, and affinity of the chemical for the vehicle (if any) are all incorporated into this subcompartment. If evaporation is occurring or if the chemical is applied in a vehicle and the vehicle has a penetration rate of its own, terms characterizing these events must be incorporated into the description, so that the concentration in the surface subcompartment, which is the driving force for penetration, can be accurately described.

The stratum corneum subcompartment represents the thin, densely packed, fully differentiated keratinocytes. This layer is the principal barrier to penetration for most chemicals due to the compactness of its lipid–protein matrix (Marzulli and Tregear, 1961; Scheuplein, 1967; Mershon, 1975; Elias and Friend, 1975; Dugard and Scott, 1984). The stratum corneum has the potential to act as a reservoir for lipophilic chemicals and may provide binding sites. There is little, if any, metabolic activity and no active transport processes (Scheuplein, 1967) associated with this lifeless layer. In this description, the stratum corneum is treated as if it were homogeneous and well stirred. This gross over-simplification will not apply for all chemicals. For other types of chemicals, it may be necessary to model the stratum corneum as the multilayered structure that it actually is (Blank and Sheuplein, 1964; Odland, 1983). Partial differential equations can be written to describe the skin if the concentration gradient within the skin is significant.

The viable epidermis subcompartment contains cells formed in the basal layer, which become keratinized and more compact as they migrate toward the surface to form the stratum corneum. The majority of the metabolic activity of the skin is found in this layer and it may provide binding sites (Marzulli et al., 1969; Pannatier et al., 1978; Finnen and Shuster, 1985). The plasma subcompartment in the skin provides blood flow to the dermis. Its vasculature is neurally regulated, provides nutrients and other essential chemicals to the skin, and affords a means for dissipation of body heat from the extremities. Pharmacokinetically, the plasma

subcompartment receives chemicals that penetrate the skin, but it also receives chemicals from arterial blood. Chemicals leave the skin via the venous blood or by metabolism. In this simplified description, the plasma subcompartment is between the viable epidermis and the dermis when, in fact, it is imbedded in the papillary dermis (Braverman and Keh-Yen, 1983; Odland, 1983).

The dermis subcompartment provides structural support for the epidermal layers above. It consists of a thick fibrous matrix of elastin and collagen and is more porous than the other compartments. Chemicals may bind to these structural components as they transit through the skin. The collagen in the dermis constitutes approximately 77% of the dry mass of the skin (Odland, 1983). The upper part of the dermis contains capillaries that provide nutrients to the viable epidermis.

The subcutaneous fat subcompartment represents a layer of variable thickness, which is poorly perfused but may provide a reservoir for lipophilic chemicals. Because it has perfusion, it could be an important compartment in its own right, even though it is below the level of the capillary beds.

Although the subcompartments make this skin compartment fairly complex for modeling purposes, it is still an obvious oversimplification of the actual intricacy of mammalian skin. Notably missing are appendages (sweat glands, hair follicles, and sebaceous glands), which have been suggested to be contributing pathways for absorption at early times with slowly diffusing electrically charged chemicals (Scheuplein, 1967; Mershon, 1975). Bookout and collaborators (1997) have described physiologically based modeling of appendages.

41.4.4 Flux Equations

Flux equations are the key to an appropriate model (see Flynn et al., 1974, for an excellent review of mass transport). The rate of change of amount (expressed as a product of volume and concentration) in a subcompartment at any time is a balance between inward flux and outward flux:

$$V\frac{dC}{dt} = \text{Influx}_{\text{total}} - \text{Efflux}_{\text{total}} \tag{41.1}$$

where V is the volume, C the free concentration (mass/volume), and Influx and Efflux are sums of the fluxes (mass/time) in each direction (Equations 41.2–41.4). The general form for the equation describing unidirectional flux where transportation of a chemical is occurring because of bulk flow of the medium is

$$\text{Flux} = QC \tag{41.2}$$

where Q is flow (volume/time) of the medium.

When the membrane between subcompartments (e.g., capillary or cell membrane) acts as a barrier to simple diffusion or when adjacent compartments such as the viable epidermis and dermis in Figure 41.4 act like there is a membrane between them, the flux from outside to inside is described by the permeability-area product and the concentration difference across the membrane:

$$\text{Flux} = PA(C_{\text{out}} - C_{\text{in}}) \tag{41.3}$$

where P is the permeability (distance/time), A the area (distance^2), and C_{out}, C_{in} the free concentrations at the outer and inner surfaces of the membrane. The thermodynamic activity differential actually drives the transport process and, if the chemicals across the barrier are in different media, it is the effective concentration at the interface that must be used in the calculation. Therefore, the concentration must be adjusted for partitioning between the media.

In some cases, movement across a barrier between subcompartments may not be by simple diffusion. If there is a saturable, active process involved, the description for flux is often represented by

$$\text{Flux} = \frac{kVC}{K_T + C} \tag{41.4}$$

where k is the maximum transport rate (mass/volume × time) and K_T the Michaelis-like transport constant (mass/volume).

41.4.5 Binding, Metabolism, and Excretion

The free concentration of a chemical in a subcompartment can also be reduced by binding to proteins or cellular macromolecules, by several types of metabolic processes, and by excretion (Lutz et al., 1980; Gerlowski and Jain, 1983). Normally, these processes are either first-order, saturable, or some combination of the two. If the process is first-order, the general equation is

$$\text{Loss} = rCV \tag{41.5}$$

where Loss has the same units as Flux (mass/time) and r is a proportionality constant (time^{-1}). This description of loss will have the same form regardless of whether the first-order loss is due to irreversible binding, metabolism, or excretion.

When the binding, metabolism, or excretion is saturable, the loss can be described by an equation of the same form as Equation 41.4 (Lutz et al., 1980; Gerlowski and Jain, 1983). The equation for saturable metabolism is

$$\text{Loss} = \frac{V_{\max}C}{K_m + C} \tag{41.6}$$

where $V_{\max}$ is the maximum reaction velocity (mass/time) and K_m the Michaelis metabolic constant.

41.4.6 Mass Balance Equations

41.4.6.1 Each Lumped Compartment

In general, for each subcompartment in Figure 41.2 a differential equation in the form of Equation 41.1 can be constructed. Equations 41.7–41.9 are for plasma, interstitial fluid, and intercellular fluid in tissues, respectively:

$$V_p \frac{dC_p}{dt} = Q_t(C_a - C_v) + P_{is}A_{is}\left(\frac{C_{is}}{R_{is/p}} - C_p\right) \quad (41.7)$$

$$V_{is} \frac{dC_{is}}{dt} = P_{is}A_{is}\left(C_P - \frac{C_{is}}{R_{is/P}}\right) + P_tA_t\left(\frac{C_t}{R_{t/is}} - C_{is}\right) \quad (41.8)$$

$$V_t \frac{dC_t}{dt} = P_tA_t\left(C_{is} - \frac{C_t}{R_{t/is}}\right) - rC_tV_t \quad (41.9)$$

where subscripts p, is, and t refer to the plasma, interstitial, and tissue (intercellular fluid) subcompartments, respectively (see Nomenclature). C_a is concentration in the arterial blood, C_v is concentration in venous blood, and R is partition coefficient between the media indicated by its subscripts. The concentration in the lumped compartment is the volume average of the concentration of the subcompartments:

$$C_i = \frac{C_pV_p + C_{is}V_{is} + C_tV_t}{V_p + V_{is} + V_t} \quad (41.10)$$

Each of the compartments in the general model shown in Figure 41.3 could require treatment as a diffusion limited lumped compartment as described in Equations 41.7 to 41.9; however, the simplification shown in Equation 41.18 will adequately describe the pharmacokinetic behavior of many lipid-soluble organic chemicals.

41.4.6.2 Skin Compartment

For skin subcompartments in Figure 41.4, Equations 41.11–41.17 account for mass fluxes within each subcompartment:

$$V_{sfc} \frac{dC_{sfc}}{dt} = P_{sc}A_{sc}\left(\frac{C_{sc}}{R_{sc/sfc}} - C_{sfc}\right) \quad (41.11)$$

$$V_{sc} \frac{dC_{sc}}{dt} = P_{sc}A_{sc}\left(C_{sfc} - \frac{C_{sc}}{R_{sc/sfc}}\right) + P_{ve}A_{ve}\left(\frac{C_{ve}}{R_{ve/sc}} - C_{sc}\right) \quad (41.12)$$

$$V_{ve} \frac{dC_{ve}}{dt} = P_{ve}A_{ve}\left(C_{sc} - \frac{C_{ve}}{R_{ve/sc}}\right) + P_pA_p\left(\frac{C_p}{R_{p/ve}} - C_{ve}\right) - \frac{V_{max}C_{ve}}{K_m + C_{ve}} \quad (41.13)$$

$$V_d \frac{dC_p}{dt} = Q_{sk}(C_a - C_v) + P_pA_p\left(C_{ve} - \frac{C_p}{R_{p/ve}}\right) + P_dA_d\left(\frac{C_d}{R_{d/p}} - C_p\right) \quad (41.14)$$

$$V_p \frac{dC_d}{dt} = P_dA_d\left(C_p - \frac{C_d}{R_{d/p}}\right) + P_{sf}A_{sf}\left(\frac{C_{sf}}{R_{sf/p}} - C_d\right) \quad (41.15)$$

$$V_{sf} \frac{dC_{sf}}{dt} = P_{sf}A_{sf}\left(C_d - \frac{C_{sf}}{R_{sf/d}}\right) \quad (41.16)$$

where the subscripts sfc, sc, ve, d, p, sf, and sk stand for surface, stratum corneum, viable epidermis, dermis, plasma, subcutaneous fat, and skin, respectively. The concentration in the skin as a whole is the volume average of the concentration of the subcompartments:

$$C_{sk} = \frac{C_{sc}V_{sc} + C_{ve}V_{ve} + C_pV_p + C_dV_d + C_{sf}V_{sf}}{V_{sc} + V_{ve} + V_p + V_d + V_{sf}} \quad (41.17)$$

It must be emphasized that these are theoretical descriptions of the process of skin penetration. These compartments have been chosen based on the current understanding of what may be the most important structural components involved. Exploration and understanding of these concepts will determine which are important subcompartments for each specific chemical to be studied.

41.4.6.3 Simplifying Assumptions

For completeness, the hypothetical compartments in Figures 41.3 and 41.4 have been relatively rigorously described using the PB-PK approach to diffusion limitation in each subcompartment; however, until methods are developed to measure the permeability-area products (PA) for the subcompartment interfaces, many simplifications must be made to make the description useful for extrapolation. One simplifying approach has been to lump P and A together into a single term, which has units of volume/time and is estimated or fit (Lutz et al., 1980; Miller et al., 1981; Angelo et al., 1984; Gabrielsson et al., 1985). A problem with the combined term is the lack of knowledge about how to scale this term so that it can be applied to another species. It has been assumed that the permeability term is related to a constant physical process across species, and the area can be scaled according to body weight (Gabrielsson et al., 1985).

There are several assumptions that have been used to collapse the subcompartments shown in Figures 41.2–41.4 and, therefore, reduce the complexity of the problem. When transfer across the cell membrane is the rate limiting step, the plasma and interstitial subcompartments can be combined into a single extracellular compartment (Lutz et al., 1980;

Gerlowski and Jain, 1983). When blood flow to the tissue is the rate limiting step (i.e., delivery of the chemical in the blood flow is much less than diffusion into the tissue), all subcompartments can be collapsed into a single well-stirred compartment where the rate of change in amount of chemical in the compartment as a whole is related to blood flow and the difference between arterial blood and venous blood concentrations (Lutz et al., 1977; Mintun et al., 1980; Andersen, 1981; Lutz et al., 1984; Matthews and Dedrick, 1984; Clewell and Andersen, 1985; Andersen et al., 1987; Leung et al., 1988; Fisher et al., 1989), which is a consolidation of Equations 41.1 and 41.2:

$$V_i \frac{dC_i}{dt} = Q_i\left(C_a - \frac{C_i}{R_{i/b}}\right) \tag{41.18}$$

where the i subscript refers to any compartment and $R_{i/b}$ is the partition coefficient between the tissue and blood. It has also been assumed that the concentration of chemical in tissue is in equilibrium with mixed venous blood. The second concentration term, tissue concentration (C_i) divided by the tissue to blood partition coefficient, is substituted for the concentration in venous blood, assuming the equilibrium condition:

$$R_{i/b} = \frac{C_i}{C_v} \tag{41.19}$$

where C_v is the concentration in venous blood leaving the tissue.

41.4.6.4 Full PB-PK Model

When differential equations are written for the skin and body compartments, they need to be connected in a way that total mass in the whole organism is conserved. The mass balance in the liver compartment is the same as Equation 41.18 except for the addition of saturable metabolism (Equation 41.6):

$$V_l \frac{dC_l}{dt} = Q_l\left(C_a - \frac{C_l}{R_{l/b}}\right) - \frac{V_{max}\dfrac{C_l}{R_{l/b}}}{K_m + \dfrac{C_l}{R_{l/B}}} \tag{41.20}$$

where C_l is the concentration in the liver. The simple skin compartment in Figure 41.3 can be described as a single well-stirred compartment with simple diffusion:

$$V_{sk} \frac{dC_{sk}}{dt} = Q_{sk}\left(C_a - \frac{C_{sk}}{R_{sk/b}}\right) + P_{sk}A_{sk}\left(C_{sfc} - \frac{C_{sk}}{R_{sk/sfc}}\right) \tag{41.21}$$

The first term on the right side of the equation describes the effect of blood flow, the second term is the net flux of chemical into the skin from the skin surface.

The concentration of chemical in mixed venous blood is the flow weighted average of all the concentrations leaving a compartment:

$$C_v = \frac{\Sigma_i(Q_i C_i)}{Q_c} \tag{41.22}$$

where Q_c is cardiac output (total blood flow).

41.4.7 Parameters of a Model

The parameters required for the model will depend on the compartments that have been chosen based on pharmacokinetics. It is important to know which parameters are available, or can be determined, because they may be the limiting factors in the structure of the model. Physiological parameters for rats with a model for volatile lipophilic chemicals (McDougal et al., 1986) are shown in Table 41.1.

It is important that the sum of the individual blood flows equals the total cardiac output. The sum of the volumes of the compartments only accounts for 91% of the body weight. The other 9% that is not accounted for is nonperfused tissue such as fur, crystalline bone, cartilage, and teeth.

Chemical-specific parameters of a model are partition coefficients, binding coefficients, and metabolic rates. Partition coefficients describe the ratio of chemical concentrations in different materials at equilibrium. They reflect the solubility of a chemical in biological fluids and tissues and are essential components of physiologically based models. Some of the partition coefficients determined by Gargas et al. (1989) that have been used for a PB-PK model of dermal absorption of organic vapors (McDougal et al., 1990) are shown in Table 41.2. These partition coefficients for volatile chemicals

TABLE 41.1
Physiological Parameters from a PB-PK Model for Rats

Lumped Compartment	Blood Flow (%Cardiac Output)	Volume (%Body Weight)
Rapidly perfused	56	5
Liver	20	4
Slowly perfused	10	65
Fat	9	7
Skin	5	10

TABLE 41.2
Partition Coefficients for Some Organic Chemicals

Chemical	Muscle/Air	Fat/Air	Liver/Air	Blood/Air
Styrene	46.7	3476	140.7	40.2
m-Xylene	41.9	1859	92.0	46.0
Toluene	27.7	1021	82.8	18.0
Perchloroethylene	20.0	1638	69.9	19.9
Benzene	10.3	499	17.8	17.8
Halothane	4.5	182	7.6	5.3
Hexane	2.9	159	12.0	2.3

TABLE 41.3
Metabolic Constants for Some Organic Chemicals

Chemical	V_{max} (mg/kg/h)	K_m (mg/L)	K_{fo} (kg/h)
Styrene	8.4	0.4	0.0
m-Xylene	4.2	0.4	2.0
Toluene	4.7	1.0	0.0
Perchloroethylene	0.0	0.0	0.3
Benzene	3.3	0.6	0.0
Halothane	7.0	0.2	0.0
Hexane	6.0	0.4	3.4

were measured by determining, at equilibrium, the ratio of concentrations in the blood or tissue to the concentration in air. Tissue/blood partition coefficients can be estimated by dividing the tissue/air partition coefficient by the blood/air partition coefficient. Jepson et al. (1994) developed a method to measure blood/saline and tissue/saline partition coefficients for nonvolatile chemicals (<1 mm Hg at 20°C) using filtration under pressure. In this case, tissue/blood partition coefficients can be estimated by dividing the tissue/saline partition coefficient by the blood/saline partition coefficient.

Metabolic constants describe the rate of loss of chemical from a lumped compartment. Table 41.3 shows saturable (V_{max} and K_m) and first-order (K_{fo}) metabolic constants for several volatile organics from McDougal et al. (1990). Most of these metabolic constants for rats were determined *in vivo* by gas uptake techniques (Gargas et al., 1986), but they can also be determined *in vitro* (Reitz et al., 1988; Sato and Nakajima, 1979; Dedrick et al., 1972). Kedderis (1997) has evaluated the extrapolation of *in vitro* enzyme induction to humans.

41.4.8 Computer Simulations

PB-PK models are sets of time dependent, nonlinear simultaneous differential equations such as those described above. Most common computer programming languages, such as FORTRAN, BASIC, and C, could be used to solve these differential equations simultaneously although the ease at which it could be done would be greatly improved with add-on integration routines and plotting packages. Continuous system simulation languages such as advanced continuous simulation language (ACSL) (AEgis Technology Group Inc., Huntsville, AL) and MATLAB® (The Math Works Inc., Natick, MA), Berkley Madona™ (Kagi, Emeryville, CA), ModelMaker (Borland®, Scotts Valley, CA), which were designed for engineering simulations, make the process of coding, debugging, modifying, and running a PB-PK model much easier. Simulation languages are interactive and allow easy data entry, on-line changes of model parameters, and plotting of the results. These simulation languages have been the most important factor influencing the increased use of PB-PK models.

41.5 HOW DO YOU DEVELOP PB-PK MODELS?

PB-PK models are unlike "canned" computer programs that can be used for various purposes once they are written. They are radically different from such multipurpose programs as statistical routines, spreadsheets, and databases because the structure of a PB-PK model is dependent on the interaction of a specific chemical with a specific species. The result of using a PB-PK model for a chemical other than that for which it was intended would be like using an Ohio state tax preparation package to prepare a California state tax return. Each unique chemical–species interaction requires that the salient physiological and pharmacokinetic principles be understood and quantitatively described. Development of a PB-PK model is an iterative process that requires insight, trial and error, and careful laboratory investigation. PB-PK models can and should be developed before the first laboratory experiment. As knowledge is gained in the laboratory, each new understanding should be quantitatively described in the model. Simulation and experimentation should be accomplished concurrently. Simulation prior to experimentation will allow appropriate data to be collected. Experimentation will confirm or increase the understanding that is quantified in the model. The key is understanding at the appropriate level as opposed to description on a superficial level.

41.5.1 Choose Compartments

Decisions about the form of the skin compartment are related to the behavior of the chemical in the skin. Lag time (the time before steady state penetration rate is achieved) is the single most important determining factor. If lag time prior to achieving steady state absorption is short, i.e., 15 min, a simple well-stirred homogeneous skin compartment (Figure 41.3) may be an adequate description. If the lag time is longer, which is more common, it will be necessary to include part or all of the skin subcompartments shown in Figure 41.4. Distribution of the chemical in the skin will determine which compartments are important to describe explicitly. Many of the methods that have been developed to study the skin will be useful for increasing the understanding required for an appropriate mathematical description. These include *in vitro* methods for metabolism and penetration, laser Doppler velocimetry, tape stripping, and ultrastructural analysis by light or electron microscopy.

Deciding which compartments, in addition to a skin, to be included in a model requires knowledge of the pharmacokinetics of the chemical of interest. Depending on the chemical, pharmacokinetic information may be available from the literature, or it may need to be determined in the laboratory before determining the structure of the model. Compartments must be included in a model to represent the major organs of metabolism and excretion. For example, a chemical that is primarily eliminated in the urine by an active process would require a kidney compartment, but a chemical that is eliminated primarily by exhalation would require a lung compartment. Metabolism studies with radiolabeled chemicals or other analytical methods, such as gas chromatography or

high-performance liquid chromatography, will provide the kinetic data required to choose the important compartments for loss of parent chemical. Additional lumped compartments must be included to account for distribution of the chemical in the animal. A lipophilic chemical would require a fat compartment or compartments while a chemical that does not distribute to the fat would not. Distribution studies with radiolabeled or nonlabeled chemicals provide the details necessary for appropriate choices of compartments. New analytical methods such as positron emission tomography or nuclear magnetic resonance imaging appear promising and may provide valuable distribution information in the whole animal. Organs in which the chemical has similar distribution may be lumped together if the organs have similar blood flow per weight of tissue. Blood flow to organs can be determined from the literature or from microsphere techniques. Other compartments that may be desired in the model would be representative of target organs for toxicity or therapeutic effect.

Decisions about the form of a lumped compartment and the requirement for subcompartments depend on the relationship between blood flow to the compartment, volume of the compartment, and solubility of the chemical in the compartment. Deciding whether the limiting factor in transfer of chemical from blood to the compartment is flow or diffusion is not always simple experimentally (Riggs, 1963). It is probably best to assume that blood flow is the limiting factor unless there is evidence otherwise or flow-limitation does not adequately describe the behavior of a compartment. The most important principle in PB-PK modeling is to use the simplest description that adequately describes the behavior of the chemical.

41.5.2 Determine Physiological Parameters

Species-specific physiological parameters, i.e., blood flow and volumes of organs, are often available in the physiology handbooks or reviews (USEPA, 1988; Fiserova-Bergerova and Huges, 1983; Gerlowski and Jain, 1983; Snyder et al., 1974) or from published PB-PK models (Adolf, 1949; Dedrick, 1973; Lutz et al., 1980; Ramsey and Andersen, 1984; Corley et al., 1990, 2000; Bouchard et al., 2001; Timchalk et al., 2002). It is necessary to make decisions about which physiological parameters to use from the literature because there will undoubtedly be a range of values available. One must avoid the temptation to change the physiological parameters to fall outside this normal range to obtain agreement between prediction and observation. If this temptation is not resisted, the result will be the loss of the ability to extrapolate. The physiological parameters in a PB-PK model for any species should be robust and not change when a different chemical is modeled, unless there is sound evidence that the chemical specifically causes changes, e.g., blood flow. When prediction and observation do not agree, there are two explanations: either the results of the experiment are not accurate or the model assumptions are inadequate. Once experimental calculations have been checked, the best approach is to determine if the structure of the model is adequate. In some cases, an important compartment has been overlooked or a diffusion limitation has been described as a flow limitation.

41.5.3 Determine Chemical Parameters

Metabolic constants, partition coefficients, and binding coefficients are much less available in the literature than the physiological parameters and they must often be determined experimentally. Metabolic constants can be determined in many ways, both *in vitro* and *in vivo*. Methods used specifically for PB-PK modeling are the tissue homogenate methods of Dedrick et al. (1972) and *in vivo* gas uptake methods for measuring metabolism of volatile chemicals (Gargas et al., 1986). Partition coefficients for volatile chemicals in blood and tissue homogenates can be determined by the vial equilibration technique (Gargas et al., 1989). Partition coefficients for nonvolatile chemicals can be determined by measuring tissue and blood concentrations after continuous dosing to achieve equilibrium. Binding, which is distinguished from partitioning because it is not linearly related to concentration, can be determined by various methods (Dedrick and Bischoff, 1968; Lin et al., 1982). The same caveat about changing physiological parameters to fit the data applies to the chemical parameters. Halving the blood/air partition coefficient because it fits the experimental results better may solve an immediate problem at the expense of the general applicability of the model.

41.5.4 Validate Model Where Absorption Is Absent

Before a PB-PK model can be used to describe the process of absorption through the skin, it is necessary to gain some confidence in the quantitative description of pharmacokinetics when absorption is absent. The model should successfully simulate blood concentrations, tissue concentrations, or expired breath after intravenous exposures at several concentrations. Urinary or fecal excretion could also be used for validation, but they are not optimum because sampling times are critical. Ideally, prolonged intravenous infusions at several concentrations and intravenous boluses at several concentrations should be used to make sure that the physiological and pharmacokinetic parameters chosen will adequately describe the processes of distribution, metabolism, and excretion for a wide range of concentrations. An alternative approach would be to achieve the same confidence with subcutaneous infusions using minipumps.

41.5.5 Validate Model with Dermal Absorption

Once the parameters not involved in absorption are fixed, then the model can be used to understand the process of absorption through the skin. Parent chemical distribution in the body after absorption through the skin and hepatic metabolism will follow the same principles independent of the absorption process. When these processes are understood and quantified, the rate of absorption through the skin can be determined based on blood, tissue, breath, or excreta concentrations.

Permeability constants can be determined by using the model to determine total chemical absorbed as long as the concentration on the skin and the surface area are known (McDougal et al., 1986).

41.5.6 Extrapolation to Humans

The ability to extrapolate from laboratory species to man is one of the most important reasons for using PB-PK models. Ramsey and Andersen (1984) have shown that a PB-PK model for inhalation of styrene vapors in rodents can predict the pharmacokinetic behavior of inhaled styrene in humans by changing the blood flows, organ volumes, and partition coefficients to those of humans. The same principles could be used to extrapolate dermal absorption studies to humans if differences in skin structure are taken into account. It will be possible to quantify the species differences in blood flow, differences in stratum corneum, epidermal and subcutaneous fat thickness and composition, as well as the effect of the type and number of appendages on skin penetration in various species.

It has been shown that organic vapor penetration rates determined in rats using a PB-PK model are two to four times greater than penetration rates in humans calculated from the literature based on the total absorbed after whole-body exposures (McDougal et al., 1990). The consistency of these comparisons suggests that differences in permeability may be due to physical differences in the skin. Using this as an example, it is important to understand some of the approaches and limitations involved in extrapolation to humans. It is not possible to directly extrapolate, with any confidence, the published PB-PK model for organic vapors in rats to the published human studies. This is because the human studies were based on urinary output and exhaled breath, and the rat studies were based on blood concentrations. It would be fairly easy to make the rat model capable of predicting exhaled breath and urinary output by adding urinary output and validating the rat model for these routes of excretion.

Once the rat model accurately predicted experimental results for urinary output and exhaled breath, the rat model could be used to address the human data by changing the physiological, pharmacokinetic, and biochemical parameters in the model to those of the human. For example, alveolar ventilation rates, blood flows, organ volumes, and urine volumes would need to be changed to those of the human. Partition coefficients, metabolic rates, and urinary excretion rates would need to be found or determined for each chemical of interest and changed in the model. With the published rat description, permeability constants were determined with confidence because the model was validated with a route where complex absorption was absent, i.e., inhalation. If the scaled-up rat model did not predict the dermal exposures in humans, it could be because the permeability constant in humans is different (as suspected) or because the physiological or pharmacokinetic parameters used for humans were incorrect. It would be necessary to make sure that these parameters were correct in humans with a route of absorption other than dermal.

Providing the rest of the description was correct, any inaccuracy in the prediction would be due to differences in permeability constant in the skin, and the permeability constant could be estimated by determining the constant required to fit the data. If the simple skin compartment (Equation 41.21) were descriptive for this chemical in the rat, it would most likely be descriptive of the same chemical in the human. Other types of chemicals, which penetrate more slowly than organic vapors, may require that the skin be broken into some or all of the subcompartments described in (Equations 41.11–41.16). In such a case, the subcompartments would also require that the structural differences in the skin between species be understood and quantified.

Other types of skin models that use first order rate constants to describe the transfer of chemicals between subcompartments have been developed. They are excellent descriptive models but do not extrapolate to other species well, because the first order rate constant is a composite of the permeability, area exposed, and the partition coefficient. These models have been reviewed and compared with PB-PK models by Roberts et al. (1999) and McCarley and Bunge (2001).

41.5.7 When the Model Fails

Paradoxically, models are often most useful when they fail to adequately describe the experimental data. During the process of developing a more adequate description of the pharmacokinetic processes involved, insight can be gained which will apply to other situations and increase the understanding of the skin, specifically, and pharmacokinetics, in general. It is the physiological foundation of the description that forces an investigator to design experiments to determine where the description is inaccurate. When frustration occurs, it is important to remember that the behavior of chemicals in living systems is not arbitrary. Chemicals and biological systems interact in accordance with physicochemical principles, which once understood are very reasonable and reliable. PB-PK modeling is an iterative process that requires theory and observation to come closer together until the final result is achieved.

41.5.8 Value of PB-PK Skin Models

PB-PK modeling can increase the understanding of the effect of vehicles on penetration rates and penetration enhancement. Jepson and McDougal (1999) showed the importance of the skin/vehicle partition coefficient by demonstrating that permeability, measured *in vivo*, could be extrapolated between different vehicles (water, corn oil, and mineral oil) with a reasonable degree of accuracy. Traditionally, flux measurements must be made on a system that is at or near steady state. Jepson and McDougal (1997) demonstrated that a PB-PK model could be used as a tool to estimate *in vivo* permeability in a situation such as an organic chemical in a

small volume of water where steady state is never achieved. These models have also been shown to accurately describe *in vitro* skin and receptor solution concentrations in the first 20 min of organic chemical in aqueous vehicle (McDougal and Jurgens, 2001). Real time breath analysis has been linked with PB-PK modeling as a tool to investigate human dermal absorption of volatile chemicals from water (Poet et al., 2000; Thrall et al., 2000) and soil (Thrall et al., 2000; Poet et al., 2002). This noninvasive approach would not be available without a PB-PK model to estimate body burden.

41.5.9 Future of PB-PK Skin Models

Improved skin compartments can be developed and validated, which include some of the subcompartments shown in Figure 41.4 to be predictive of penetration rates of chemicals that have more complicated absorption profiles. Pharmacodynamic models that quantitatively describe the molecular events that occur in the skin with local toxicity (for example, psoriasis, contact dermatitis, or skin cancer) can be developed. These models might describe tissue levels, production, and turnover rates of important proteins, signaling molecules, and mRNA levels that are responsible for deleterious changes in skin function. With appropriate biologically based models, it is possible to make the connection between amount of chemical on the surface and the therapeutic or toxic effect. When validated, these validated models could be applied to the development of biomarkers and prophylaxis.

41.6 CONCLUSION

Physiologically based pharmacokinetic models provide tremendous capacity to increase the understanding of skin absorption and the effects of chemicals in the skin. The ability to extrapolate between *in vivo* exposure conditions, doses, and species allows laboratory animal studies to provide a wealth of information applicable to human exposure situations that can be used for exposure and risk assessment. The ability to apply quantitative descriptions to processes occurring in the skin is limited only by our ability to understand the processes involved.

NOMENCLATURE

C	Concentration (mass/volume)
V	Volume
A	Area (distance^2)
Q	Flow (volume/time)
P	Permeability (distance/time)
K_m	Michaelis metabolic constant (mass/volume)
K_T	Michaelis-like transport constant (mass/volume)
K	Maximum transport rate (mass/volume $\times$ time)
R	Proportionality constant (time^{-1})
R	Partition coefficient (unitless, ratio of concentrations)
V_{max}	Maximum velocity (mass/time)

Subscripts

a	Arterial
b	Blood
c	Cardiac output
d	Dermis
e	Extracellular
i	Ith tissue compartment
is	Interstitial
p	Plasma
sc	Stratum corneum
sf	Subcutaneous fat
sfc	Surface
sk	Skin
t	Tissue
v	Venous
ve	Viable epidermis

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42 Methods for *In Vitro* Skin Metabolism Studies

Robert L. Bronaugh

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42.1 INTRODUCTION

It has been known for years that enzymes in skin catalyze a wide variety of metabolic reactions (Pannatier et al., 1978; Bickers, 1980; Kappus, 1989). All the major enzymes important for systemic metabolism in the liver and other tissues have been identified in skin (Pannatier et al., 1978). Often enzyme activity has been found to be lower in skin (on a per mg tissue basis) when compared to the liver (Bronaugh et al., 1989; Mukhtar and Bickers, 1981). However, the skin is the largest organ in the body with a surface area of 2 m^2 and total weight estimated at 4 kg—about three times that of the liver (Pannatier et al., 1978). Therefore, the skin can play an important role as a portal of entry of chemicals into the body.

Some chemical groups such as esters, primary amines, alcohols, and acids are particularly susceptible to metabolism in skin. Many esters are hydrolyzed by esterase to their parent alcohol and acid molecules (Boehnlein et al., 1994; Kenney et al., 1995). Primary amines are frequently acetylated during percutaneous absorption through skin (Nathan et al., 1990; Kraeling et al., 1996; Yourick and Bronaugh, 2000). Oxidation/reduction and conjugation of alcohols and acids are commonly observed in skin (Nathan et al., 1990; Boehnlein et al., 1994).

Chemicals that undergo significant metabolism in skin may exhibit greater or lesser biological activity than predicted simply from skin penetration studies. A more thorough examination of the safety or efficacy of these compounds can be determined by evaluating skin absorption and metabolism simultaneously using *in vitro* techniques.

42.2 REASONS FOR DOING *IN VITRO* STUDIES

Skin metabolism studies are difficult to accurately conduct *in vivo* because of systemic metabolism that takes place before samples are collected in the blood, urine, or other site. *In vitro* studies isolate the skin from the metabolic activity in the rest of the body. When studies are conducted using viable skin in diffusion cells, metabolites can be measured in skin homogenates or in the receptor fluid directly beneath the skin. Also, *in vitro* studies may be the only ethical way to obtain human skin metabolism data for chemicals with safety concerns.

42.3 MAINTENANCE OF SKIN VIABILITY IN DIFFUSION CELLS

The assembly of skin in diffusion cells is described in general terms in Chapter 34. This chapter discusses methods for maintaining viable skin in metabolism studies.

Human or animal skin should be freshly obtained. Skin previously frozen for shipping or storage is unsuitable for metabolism studies. Enzyme activity with some stable enzymes can sometimes be still observed in nonviable skin but the activity may be at a reduced level as observed for esterase activity (Boehnlein et al., 1994; Kenney et al., 1995).

The viability of rat skin was maintained for at least 24 h in flow-through diffusion cells using several physiological buffers as the receptor fluid (Collier et al., 1989). For 24 h studies, the use of flow-through cells is required likely, so that nutrients are continually provided to the skin.

Although a tissue culture media (minimal essential media [MEM]) was satisfactory in maintaining skin viability, it was not required. Simpler balanced salt solutions such as HEPES-buffered Hanks' balanced salt solution (HHBSS) or Dulbecco-modified phosphate-buffered saline worked just as well and are potentially less problematic for analytical reasons. Some of the vitamins, cofactors, and amino acids contained in MEM absorb in UV light and can interfere with UV detection during HPLC analysis. Bovine serum albumin (BSA) has been added to the receptor fluid to enhance the

partitioning of lipophilic test compounds from skin into the receptor fluid.

42.4 SKIN VIABILITY ASSAYS

Viability of skin was primarily assessed in our initial studies by measuring aerobic and anaerobic glucose utilization (Collier et al., 1989). Anaerobic metabolism of glucose to lactic acid predominates in skin and so this assay has been commonly used. Glucose is the primary energy source for skin cells and has been monitored by tissue banks to assess skin viability for transplants (May and DeClement, 1981). We used electron and light microscopy techniques to assess viability by demonstrating that the cellular organelles were still intact at the end of 24 h studies. Skin metabolism of estradiol and testosterone was also maintained for 24 h.

We have observed that the addition of 4% BSA to HHBSS results in a lowering of lactate levels measured in the skin viability assay (Hood and Bronaugh, 1999). Therefore, the 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetra-zolium bromide (MTT) assay was adapted to assess skin viability when BSA was required in the receptor fluid. The MTT assay of skin viability was not affected by addition of BSA to the receptor fluid. The viability of human, fuzzy rat, and hairless guinea pig skin was found to be maintained for 24 h. However, the assay can only be conducted at the end of a study when skin can be removed from the diffusion cell. The lactate measurement of glucose utilization can be conducted during the course of an experiment.

42.5 SKIN METABOLISM DURING *IN VITRO* ABSORPTION STUDIES

Early studies from our laboratory used intact viable dermatome skin sections from mice, rats, hairless guinea pigs, and humans in flow-through diffusion cells to study the penetration and metabolism of estradiol and testosterone (Collier et al., 1989), acetyl ethyl tetramethytetralin (AETT) and butylated hydroxytoluene (BHT) (Bronaugh et al., 1989), benzo[a]pyrene and 7-ethoxycoumarin (Storm et al., 1990), and azo colors (Collier et al., 1993).

The percutaneous absorption and metabolism of three structurally related compounds, benzoic acid, *p*-aminobenzoic acid (PABA), and ethyl amino-benzoate (benzocaine), were determined *in vitro* with hairless guinea pig and human skin (Nathan et al., 1990). Approximately 7% of the absorbed benzoic acid was conjugated with glycine to form hippuric acid. Acetylation of primary amines was found to be an important metabolic step in skin. For benzocaine, a molecule susceptible to both *N*-acetylation and ester hydrolysis, 80% of the absorbed material was acetylated, while less than 10% of the absorbed ester was hydrolyzed. PABA was much more slowly absorbed than benzocaine and was also less extensively *N*-acetylated. Acetyl-PABA was found primarily in the receptor fluid at the end of the experiments, but the receptor fluid contained only 20% of the absorbed dose. Much of the absorbed PABA remained unmetabolized and in the skin as might be expected for an effective sunscreen agent. The compound in the skin would probably not have been exposed to *N*-acetylating enzymes if it was localized primarily in the stratum corneum. A similar pattern of benzocaine metabolism was observed in human and hairless guinea pig skin; however, there appeared to be less enzyme activity in human skin.

The effect of benzocaine dose on its absorption and metabolism was determined in the hairless guinea pig (Kraeling et al., 1996). It was of interest to determine if metabolism of absorbed benzocaine remained extensive when the radiotracer doses used in our earlier studies were increased to doses simulating human use conditions as a local anesthetic. Percutaneous penetration of benzocaine increased 50-fold when the applied dose increased from 2 to 200 μg/cm^2. Metabolism of benzocaine to acetylbenzocaine was reduced at the higher dose but still, approximately, one-third of the absorbed dose was metabolized (Table 42.1). The metabolism of benzocaine in skin may not affect the local anesthetic activity of a topical commercial product since benzocaine and acetylbenzocaine were found to have similar potencies in reducing conductance in the isolated squid giant axon (Kraeling et al., 1996).

Esterase activity and alcohol dehydrogenase activity were characterized in hairless guinea pig skin with the model compounds methyl salicylate and benzyl alcohol (Boehnlein et al., 1994). Subsequently, the absorption and metabolism of the cosmetic ingredient retinyl palmitate was determined in human and hairless guinea pig skin.

TABLE 42.1
Effect of Benzocaine Dose on its Metabolism; Percentage Distribution of Benzocaine and Metabolites in Receptor Fluid and Skin in 24 h

Location and Compound	Dose Level		
	2 μg/cm^2	40 μg/cm^2	200 μg/cm^2
Receptor fluid			
Benzocaine	9.6 ± 4.2	50.7 ± 6.6	54.0 ± 5.2
AcBenz	83.8 ± 4.4	43.8 ± 5.7	37.9 ± 3.6
PABA	1.0 ± 0.3	0.1 ± 0.1	0.9 ± 0.5
AcPABA	5.1 ± 1.0	5.8 ± 0.9	7.2 ± 1.7
Skin			
Benzocaine	26.7 ± 14.2	2.4 ± 2.4	62.7 ± 12.2
AcBenz	6.9 ± 6.9	34.4 ± 20.3	20.9 ± 11.7
PABA	4.3 ± 4.3	3.2 ± 3.2	1.5 ± 1.3
AcPABA	24.7 ± 14.9	15.2 ± 16.0	14.9 ± 1.2
Total			
Benzocaine	10.7 ± 3.3	49.9 ± 6.5	57.3 ± 3.7
AcBenz	80.5 ± 3.8	43.6 ± 5.6	34.3 ± 3.4
PABA	1.4 ± 0.2	0.1 ± 0.1	0.9 ± 0.5
AcPABA	6.5 ± 1.0	5.9 ± 1.0	7.6 ± 2.0

Note: Values are the mean ± S.E. for 1–6 determinations in each of the three animals. The 40 μg/cm^2 dose level values are the mean ± S.E. of 2–3 determinations in each of the four animals. The dosing vehicle was acetone. AcBenz = acetylbenzocaine; PABA = *p*-aminobenzoic acid; and AcPABA = acetylPABA.

The metabolism of methyl salicylate was determined in viable and nonviable hairless guinea pig skin. In viable skin over 50% of the absorbed compound was hydrolyzed by esterases in skin to salicylic acid. Twenty-one percent of the absorbed compound was further conjugated with glycine to form salicyluric acid. Greater esterase activity was observed in male skin. Esterase is a stable enzyme and hydrolysis of methyl salicylate also occurred in nonviable skin. However, no conjugation of salicylic acid was observed in nonviable skin.

Oxidation of benzyl alcohol was also observed in hairless guinea pig skin. Approximately 50% of the absorbed benzyl alcohol was oxidized to benzoic acid in viable skin with a small portion of this compound being further metabolized to the glycine conjugate—hippuric acid. As with the ester, significant activity was also observed in nonviable skin and greater oxidation of the alcohol was obtained with male skin.

The absorption and metabolism of retinyl palmitate was measured to see if ester hydrolysis and alcohol oxidation occurred with this cosmetic ingredient. Most of the absorbed radioactivity remained in the skin. A substantial amount of the absorbed compound was hydrolyzed to retinol but no oxidation of the alcohol to retinoic acid was observed. Any effects of retinyl palmitate on the structure of skin may be due to the formation of retinol during percutaneous absorption.

Absorption values from *in vitro* studies with viable hairless guinea pig skin have been found to compare closely with *in vivo* results for phenanthrene (Ng et al., 1991) and for pyrene, benzo[a]pyrene, and di(2-ethylhexyl) phthalate (Ng et al., 1992). Also, significant metabolism was observed *in vitro* during the absorption of all four compounds.

Phenanthrene was metabolized *in vitro* to 9,10-dihydrodiol, 3,4-dihydrodiol, 1,2-dihydrodiol, and traces of hydroxy phenanthrenes (Ng et al., 1991). Following topical administration of phenanthrene, approximately 7% of the percutaneously absorbed material was converted to the dihydrodiol metabolites.

Numerous metabolites of benzo[a]pyrene were formed during percutaneous absorption through hairless guinea pig skin (Ng et al., 1992). Of particular interest was the identification of benzo[a]pyrene 7,8,9,10 tetrahydrotetrol in the diffusion cell receptor fluid. This metabolite is the hydrolysis product of the ultimate carcinogen, 7,8-dihydroxy, 9,10-epoxy-7,8,9,10-tetrahydro-benzo[a]pyrene. This study demonstrates that skin metabolism is likely responsible for skin tumors formed following topical benzo[a]pyrene administration. In the earlier phenanthrene study (Ng et al., 1991), no known carcinogenic metabolites were formed during skin permeation. This finding is consistent with the lack of tumorigenicity of phenanthrene in rodents.

Since the systemic toxicity of topically applied compounds is sometimes evaluated by the oral route of administration, the effect of route of administration on metabolism of (^{14}C) 2 nitro-*p*-phenylenediamine (2NPPD) was examined *in vitro* in the fuzzy rat (Yourick and Bronaugh, 2000). Rat skin dermatomed to approximately 250 μm and full thickness rat intestinal tissue (from the jejunum) were assembled into flow-through diffusion cells perfused with HHBSS to maintain viability. 2NPPD was applied for 30 min to skin in a semipermanent hair dye formulation and to intestine in HHBSS (pH 6.5). Similar amounts of radioactivity penetrated into the receptor fluid from each tissue during the 24 h studies.

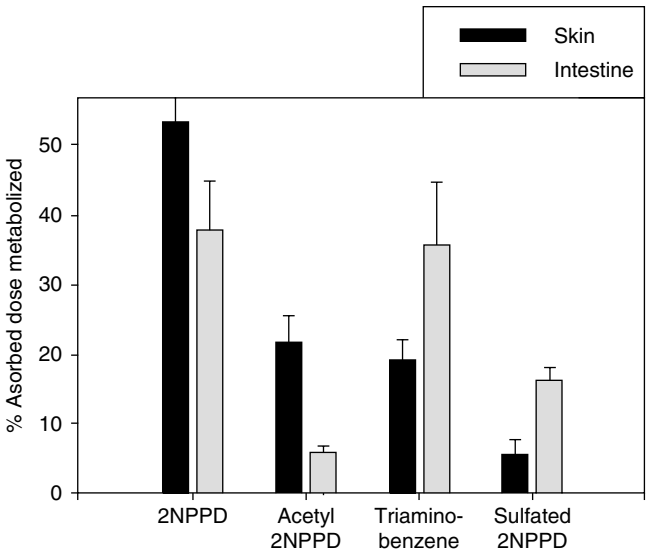


FIGURE 42.1 Metabolism of 2NPPD during absorption through rat skin and intestinal tissue. Values are the mean ± S.E. of three individual rat studies ($n = 3$).

The metabolism of 2NPPD was determined in receptor fluid fractions using an HPLC method. More than 50% of the 2NPPD applied to skin remained unmetabolized while only 40% of 2NPPD was unmetabolized by intestine (Figure 42.1). Substantially more acetylation of 2NPPD to N4-acetyl-2NPPD occurred during absorption through skin. However, triaminobenzene was formed to a greater extent in intestine. The amount of sulfated 2NPPD or metabolites (actual compound or compounds not determined) was also greater in effluent from intestinal tissue. The extent of metabolism of 2NPPD in human skin (semipermanent hair dye vehicle) was also determined. Approximately 60% of the absorbed radioactivity was metabolized to equal amounts of triaminobenzene and N4-acetyl-2NPPD. No sulfated compounds were found in effluents from human skin. These studies showed significant differences in metabolism during penetration through human and rat skin, as well as differences in metabolism through rat skin and intestinal tissue.

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43 Predictive Toxicology Methods for Transdermal Delivery Systems

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Predicting potential toxicologic responses to transdermal delivery is a complex procedure, involving both traditional toxicology protocols for evaluating results of systemic exposure and topical studies assessing skin–drug interactions and reactions. Evaluation of individual drug and system components is followed by final system testing to assess possible interactions. Risk is estimated by analyzing toxicologic data quantitatively, with estimation of human exposure based on dose–response extrapolations. Formulation or system changes designed to minimize risk are evaluated. In some instances, local intolerance to a compound—either due to irritation or sensitization—may preclude development of a transdermal product despite efficacious plasma levels. For viable projects with acceptable toxicologic profiles, a strategy is implemented to manage the risk of irritation and sensitization. In addition to the usual drug-specific systemic toxicology and regulatory issues, a toxicology evaluation plan for transdermal dosage forms must include primary and cumulative irritation and sensitization testing. The plan must also take into account the U.S. Food and Drug Administration's (FDA) categorization of new transdermal systems and novel excipients as new chemical entities (NCEs), subject to standard nonclinical testing procedures.

43.1 TOXICOLOGY EVALUATION PLAN

Development of a toxicology evaluation plan for a transdermal system involves two major stages: assessment of previous experience with both the drug(s) and system components, and selection of necessary and appropriate tests. Assessment of previous experience includes review of data in the literature and in regulatory submissions available through the Freedom of Information Act (1996). Substantial published knowledge of the drug allows analysis of the effects of various doses, plasma drug levels, and regimens, and generally reduces testing requirements. Information about oral, intravenous, and especially subcutaneous administration may be used for comparison or to support the safety and efficacy of transdermal delivery when equivalent plasma levels are achieved and no significant biotransformation occurs. Literature and other available data about compounds previously cleared by the FDA may be useful in determining areas for additional study. Similarly, evaluations of proposed system materials in the literature and previous FDA submissions may provide valuable information about individual system components or possible interactions of materials.

An additional factor in the evaluation plan is the categorization by the FDA of new transdermal systems as NCEs, subject to standard nonclinical testing procedures. A complete toxicology profile—including subchronic, chronic, carcinogenic, and genotoxic assays—is required for all compounds delivered transdermally. This requirement may be reduced when systems incorporate a compound already marketed in another dosage form. Transdermal systems that achieve lower plasma drug levels than other marketed forms of the drug and transdermal systems that incorporate drugs or components previously evaluated for irritation or topical sensitization may require few nonclinical studies. Additional toxicology studies for topical products may include photosafety studies and irritation and sensitization studies (see the following discussion) with the components.

The pharmacokinetic analysis of plasma drug levels in an acceptable animal model is recommended prior to clinical use of the transdermal system. This will not only support

the proposed toxicology plan, but also, because transdermal systems typically contain much more drug than will be delivered, provide safety data to the FDA that there is controlled delivery of drug. Some divisions of the FDA have required the demonstration that no immediate release or higher than anticipated drug delivery rates were observed from any of the systems tested, and only a fraction of the total drug content in the patch was delivered over the wearing period.

Toxicokinetic analysis—pharmacokinetic analysis applied to dose-ranging and definitive toxicity testing—is increasingly important to determine drug exposure in nonclinical toxicology protocols for transdermal and other dosage forms, and is included in guidelines drafted by the International Conference on Harmonisation (Federal Register, 1995). Toxicokinetic studies describe the relationship between systemic exposure, dose administered, and time, providing data to support safety claims at various doses. Results of these studies contribute to the design of subsequent nonclinical studies. (For additional details about toxicokinetic studies, see Dixit, 2006 and Kantrowitz and Yacobi, 1994.) Because transdermal delivery of drugs bypasses the first-pass effect, the drug-to-metabolite ratio changes. If a drug has an active major metabolite, measurement of both the parent and major metabolite may be indicated during the pharmacokinetic/toxicokinetic analysis.

Studies of skin metabolism may also be required. Although transdermal administration bypasses hepatic circulation, molecules absorbed through the skin will come into contact with the skin's metabolic system (Hikima et al., 2005). (For a comprehensive review of the skin's enzymatic activity, see Baron and Merk, 2001; Steinstrasser and Merkle, 1995; Noonan and Wester, 1985.) Skin metabolism may alter the delivery profile and pharmacological effects of some percutaneously absorbed compounds (Boehnlein et al., 1994; Guy and Hadgraft, 1982; Hashiguchi et al., 1998; Tang-Liu et al., 1999; see also Chapter 9 of this book). The effects of first-pass skin metabolism of transdermally delivered compounds can be predicted by *in vitro* biotransformation studies (Brand et al., 2000; Cormier et al., 1991; Friedberg, 1998; Sintov et al., 2002; Smith et al., 2000). The toxicology profile of compounds may be altered by the quantitative modification of metabolic pathways caused by skin metabolism (Cleary et al., 1984; Bucks, 1984). These changes, which are sometimes measured by comparison with oral or intravenous data, may result in local irritation, sensitization, or adverse systemic effects. Changes resulting from skin metabolism may also transform inactive prodrugs with favorable permeation characteristics into metabolically active parent drugs (Sintov et al., 2002; Imoto et al., 1996, Tauber, 1989). Skin metabolism and subsequent formation of protein conjugates may assist in prediction and evaluation of skin sensitization assays (Dimitrov et al., 2005; Wilson et al., 2003; Divkovic et al., 2003, 2005; Gerberick et al., 2004). In addition, genetic variations in the skin's enzymatic activity may account for differences in therapeutic efficacy among patients. Generally, however, the effect of the skin's first-pass metabolism is slight since skin cells have lower intrinsic metabolic activity than hepatocytes, and only a few cell layers within a limited area are perfused by a transdermally delivered compound. For example, skin metabolism of propranolol, which is metabolized extensively by the liver, was almost negligible (Cormier et al., 1991).

Phototoxicity issues may also need to be addressed with transdermal drug delivery. Although phototoxicity testing is conducted primarily with humans, both the FDA and the Organisation for Economic Co-operation and Development (OECD) have draft guidelines for photosafety testing (including phototoxicity, photoallergy, photogenotoxicity, photocarcinogenicity), which provide decision trees to assist in the evaluation. According to the OECD, photosafety testing is indicated if the drug absorbs light between 270 and 700 nm on the ultraviolet (UV)-visible light absorption spectrum, and if the drug is applied topically or accumulates in light-exposed areas. The OECD considers the *in vitro* 3T3 neutral red uptake (NRU) phototoxicity assay validated and provides a test guideline. This assay can be used to evaluate the drug substance, but other testing may be required on the finished drug product. If additional *in vivo* testing is warranted, a controlled clinical trial is suggested. The FDA has stated that testing only the drug substance may be acceptable, but that the drug product (final formulation) should be tested if the product absorbs light between 290 and 700 nm, as well as if the product is "applied to the skin or eyes, or persist or accumulate in one of these areas, or 2) are known to affect the skin or eyes." The FDA does not specify how to test for photosafety, but acknowledges the use of *in vitro* studies, *in vivo* nonclinical studies, and human clinical studies, and encourages "the submission of specific data that may help in evaluating the regulatory acceptance of such assays." The interagency coordinating committee on the validation of alternative methods (ICCVAM) is expected to evaluate the 3T3 *in vitro* assay, but no information is available as of this writing. The communication of risk is key; factors to consider include the half-life, acute or chronic use, pharmacologic class, and persistence in the skin (especially in areas exposed to sunlight). If the risk is sufficient, additional tests may evaluate photoallergy, photogenotoxicity, and photocarcinogenicity.

Polymers and permeation enhancers used in transdermal systems need to be evaluated for potential adverse effects before testing the drug delivery system in humans. Polymers used in transdermal drug delivery systems may not penetrate the skin themselves, but components of the polymer, such as residual monomers, reactive agents, or processing additives, may migrate. The effects of polymers or extracts will depend on unique chemical characteristics and the amount (or dose) of polymer/extract administered. To assist in the selection of materials, literature from the manufacturer and data from published studies should be reviewed for clinical and nonclinical safety information (Venkatraman et al., 2000).

The polymer and extracts should be biocompatible since they are in contact with components that penetrate the skin. If necessary, *in vitro* and *in vivo* tests are conducted to evaluate the biocompatibility of the polymer or extract and of the drug delivery system and thus to assess the safety of the system. *In vitro* studies should be conducted before initiating *in vivo*

studies. The tests listed in the International Standards Organization/American National Standards Institute/Association for the Advacement of Medical Instrumentation (ISO/ANSI/AAMI) Standard 10993, Biological Evaluation of Medical Devices, are intended to provide testing strategies for medical devices. There are similar guidelines in the U.S. Pharmacopeia (USP) 29—National Formulary 24 for plastics used as drug containers. These tests may also be used to test biocompatibility of polymers used in transdermal drug delivery systems.

Extracts may also be tested for the potential to cause topical sensitization. Preparation of polymer extracts is defined by guidelines, but the choice of conditions should come close to the conditions of manufacture of the drug delivery system. Extracts are evaluated using *in vitro* cytotoxicity tests as well as *in vivo* irritation, intracutaneous injection, systemic injection, and implantation studies. A sample of the polymer may also be evaluated in these *in vivo* tests. The final transdermal drug delivery system must also be evaluated in standard nonclinical toxicology studies to evaluate safety of the system.

If there is systemic absorption of a polymer or a permeation enhancer that is not an NCE, manufacturer information and published scientific literature should be reviewed. If data indicate that blood levels are acceptable based on historical exposure or existing toxicology data, then conducting irritation and sensitization studies with the final formulation and providing a written review and justification of the use of the polymer or permeation enhancer may be all that is necessary.

If the polymer or permeation enhancer is released systemically and is an NCE, the FDA Guidance for Industry: Nonclinical Studies for Development of Pharmaceutical Excipients should be reviewed. The new excipient may require a full toxicology package consisting of acute, chronic, reproduction, and carcinogenicity testing to establish safe limits for the compound. The FDA will consider reduced programs based on the availability of adequate prior human exposure and short durations of clinical use.

New adhesives should also be tested for biocompatibility. Extracts may be evaluated using *in vitro* cytotoxicity tests and *in vivo* irritation, intracutaneous injection, systemic injection, and implantation studies. Placebo transdermal systems may be tested in irritation studies to evaluate the contribution of the adhesive to irritation. Sensitization studies should always include placebos to differentiate reactions between the drug and the excipients.

Beyond these general toxicology considerations, irritation and sensitization tests are a primary focus for evaluating a transdermal dosage form.

43.2 IRRITATION

43.2.1 *In Vivo* Irritation Testing

The New Zealand white rabbit or guinea pig (Hartley or hairless strains) are the most often used species to test transdermal systems for irritation, but the pig (including mini- and microstrains) have also been used. Evaluation of the primary irritation potential of transdermal systems should be tested prior to single application in humans, including prototypes and the final formulation. Evaluation of the cumulative irritation potential of transdermal systems should be tested prior to multiple applications in humans.

Primary irritation (one application of a transdermal system irrespective of duration of wear) is most often tested by modifications of the original Draize test. Draize type tests have been used by several government bodies, including the department of transportation, the consumer product safety commission, the environmental protection agency, and the OECD (Patrick and Maibach, 1994). Transdermal systems should be applied for the intended dosing interval in humans (e.g., for a 7-day transdermal system, apply for 7 days).

Additional tests on the components of a transdermal system, as well as on the final formulation and placebo, may include a cumulative/subchronic irritation study (more than one application, each for the intended clinical wear) and dermal toxicity study (Robinson and Perkins, 2002; Steinberg et al., 1975). The duration of subchronic irritation studies should be confirmed with the FDA, but is typically 28 days (e.g., four applications of a weekly application) or 90 days in duration, depending on the active ingredient and the intended use. Plasma concentrations are determined on the first and last day to substantiate systemic exposure during these studies (28–90 days). Release of drug from the transdermal system may also be determined by measuring residual drug in used systems. For both active and placebo systems, erythema and edema of the skin sites are also monitored, and clinical pathology and histology observations are made to establish dose-related drug effects. Here again, the system should be applied for the intended dosing interval in humans. Typically, transdermal systems are not applied to the same skin site on people, and therefore, applications should be rotated among two to four skin sites on the toxicology animal model (guinea pigs, rabbits, pigs). Comparative testing using a novel human 4 h patch has also been proposed for acute skin irritation testing (Basketter et al., 2004; Robinson et al., 2005).

43.3 SENSITIZATION

Skin sensitization, or contact sensitization, is a delayed type immune response mediated by T cells. Each transdermal system formulation should be tested in the guinea pig model prior to multiple applications in humans.

Numerous *in vivo* tests and strategies are available, developed, and have been adopted by several regulatory agencies to identify potential skin sensitizers (Basketter et al., 1995; Gerberick et al., 2000; Kimber et al., 2001a,b; Maurer et al., 1994; Ryan et al., 2000, 2001). Each transdermal system formulation should be tested in the guinea pig model prior to multiple applications in humans. The European Economic Community (EEC) suggests the guinea pig maximization test as the first test, based on the original literature and its validation with moderate or weak allergens.

43.3.1 Guinea Pig Model

The guinea pig is the animal most frequently used to evaluate the potential for transdermal systems to produce contact sensitization. The hairless guinea pig is also used as a predictive model to study irritation and sensitization. Hairless guinea pigs are derived from a natural mutation, are euthymic, and are hairless except for continuous growth of hair at the nose and feet. The hairless guinea pig strain offers a cost-effective alternative to the Hartley guinea pig strain, since depilation or shaving is not required. In addition, undue irritation during removal of bandages is reduced, and application sites are not obscured by hair growth during scoring. Hairless guinea pigs have been evaluated in primary skin irritation and sensitization studies (Buehler and Kreuzman, 1990; Chester et al., 1988).

Delayed contact hypersensitivity testing of transdermal delivery systems must not underpredict potential toxic effects. Protocols designed to test products that wash off the skin may not be optimal for systems that deliver drugs through the skin. The sensitization potential of the drug is assessed early in the development program, before the development of the transdermal product. This evaluation of the inherent sensitization potential of the compound may be done by intradermal injection models; for evaluation of compounds with good transdermal flux, intermediate formulations, or final formulations, a modified Buehler protocol may be used (Tsuchiya et al., 1982). This modified protocol may use five or more 24-h occlusive applications of materials over a 3-week period (Chester et al., 1988). Topical applications can be augmented by Freund's Complete Adjuvant (FCA) injections, with or without drug, to the shoulder region. Treatment sites for the first and last inductions are scored for erythema/edema at 2 and 24 h after removal of the test article. Approximately 2 weeks after the last topical application of the induction period, all groups are challenged with 24-h occlusive topical application of appropriate test and control formulations.

Modifications of these standard methods have been adopted to test transdermal systems. For example, transdermal systems are applied three times a week for 3 weeks. The protocol should include at least three groups: one group of at least 10 guinea pigs induced with the placebo formulation (final formulation with permeation enhancers if appropriate, but no drug), one group of 10 guinea pigs induced with the final formulation with drug, and a positive control group. The positive control group animals are typically induced with a topical application, not a transdermal system, of a known moderate to severe sensitizer to demonstrate that the animals can mount a sensitization response. This group may require only five animals. As with topical applications, transdermal applications can be augmented by FCA injections, with or without drug, to the shoulder region. After a rest period of 2 weeks, each animal in the transdermal group is challenged with both the placebo and active systems. This will differentiate any sensitization reactions to drug from those to excipient(s). If the intended dosing interval of the transdermal system is greater than 24 h, the design of sensitization studies may be modified, for example, each induction application may be 48 h instead of 24 h. If a second challenge is needed to confirm responses, the transdermal animals can be rechallenged 2 weeks after the first challenge. Challenges may include intradermal injections of the drug or each excipient/permeation enhancer to differentiate the causative compound. For each additional challenge, the challenge materials should be applied in the same manner to an additional group of five naïve guinea pigs to act as an irritation control group. For all challenges, skin sites are evaluated at 2, 24, and 48 h (or longer if indicated) after the test compound or the transdermal system is removed. The scoring system remains the same, and a reaction is generally considered positive when a combined erythema and edema score of 2 or greater is still present 48 h after removal of the system. Sensitization potential is characterized based on the number of animals exhibiting reactions (0–8% [weak] to 81–100% [extreme]).

43.3.2 Murine Models

When testing a component and not the transdermal system, murine models such as the Mouse Car Sensitivity Test (MEST) and Local Lymph Node Assays (LLNAs) have objective endpoints and allow evaluation of pigmented materials. A quantitative relationship among LLNA, guinea pig, and human skin sensitization assays has been established (Yamano et al., 2005; Schneider and Akkan, 2004; Basketter et al., 2005). Murine assays also reduce cost, test duration, number of animals used, and required care space.

43.3.3 Regulatory Position

In 1992, both the OECD and EEC reduced the number of primary recommended tests and accepted primarily the Buehler and the maximization tests; the EEC favors the maximization test as the first test (Maurer et al., 1994). It was recommended that tests be based on the original literature. In addition, validation should occur with moderate or weak allergens. Other validated protocols are acceptable in certain cases, and the OECD accepts the MEST and LLNA as screening tests. Positive results in these *in vitro* tests are sufficient, but negative results in mouse tests must be confirmed in guinea pig tests.

43.4 SUMMARY

The results of animal sensitization studies must be related to potential human hazards. Unfortunately, human populations show greater variability than animal test systems. This is particularly problematic because a negative sensitizer in the test does not guarantee that the compound will not sensitize humans. For example, standard predictive tests in guinea pigs indicated that clonidine was not a sensitizer. This observation was confirmed in clinical trials. Following introduction in the marketplace, a large number of users (19%) became sensitized to transdermal clonidine after prolonged use. Conversely, a weak sensitizer with good potential utility for therapy may not need to be abandoned, but may need to be patch tested in humans. This chapter describes toxicity

tests used for establishing the safety of a transdermal delivery system. In addition to sensitization and irritation potentials, standard preclinical testing procedures are required for any new drug to be approved for marketing. In the United States, the FDA treats all new transdermal dosage forms as NCEs; however, a transdermal system incorporating a drug or compound already approved in a different dosage form may have a swifter approval process. Systemic safety may not be an issue if the dosage for drugs to be delivered transdermally produces plasma levels far lower than those produced by marketed forms of the same drug. If systemic safety has already been adequately demonstrated, nonclinical studies, beyond evaluation of acute and cumulative irritation and sensitization, may be minimal or not required.

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44 Animal, Human, and *In Vitro* Test Methods for Predicting Skin Irritation

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44.1 INTRODUCTION

Contact with external irritating agents, such as dishwashing liquid, enzymes, or raw meat, can result in irritant contact dermatitis (ICD), a localized nonimmunologic condition. ICD ensues when irritant stimuli overpower the defense and repair capacities of the skin (Goldner and Jackson, 1994; Walle, 2000). Exposure to potent irritants or exposure to mild irritants for an extended period of time will increase the likelihood of developing ICD.

Preventive measures, including the utilization of proper skin care, the avoidance of harsh soaps, and the use of protective garments such as gloves, will decrease the risk of irritant dermatitis occurring. In addition, it is of crucial importance to test the irritant potential of any substance that will be applied to human skin, so that its likelihood of inducing irritant dermatitis is known. Federal regulatory agencies require toxicity testing to determine the safety or hazard of various chemicals and products prior to human exposure. This information is used to properly classify and label products according to their potential hazard (Bashir and Maibach, 2000).

No one assay is able to accurately portray irritation in its entirety. This is because irritant dermatitis may result from either acute or cumulative injury, and may involve inflammation or skin necrosis (corrosive). A number of animal, human, and *in vitro* test methods have been developed, each portraying some but not all aspects of irritation. Each model has its unique benefits and limitations.

44.2 ANIMAL MODELS

44.2.1 Draize Rabbit Assay

To evaluate primary irritation and corrosion, the Draize animal model or one of its modifications is utilized. The Draize rabbit test was developed in 1944, and has since been adopted in the U.S. Federal Hazardous Substance Act (FHSA) (code of FR) (Patrick and Maibach, 1989). The test involves two (1 in.^{2}) test sites on the dorsal skin of six albino rabbits. One site is abraded (through use of a hypodermic needle across the rabbit skin) and the other site remains intact. The stratum corneum is broken on the abraded site, without loss of blood. The undiluted "irritant" materials (0.5 g for solids or 0.5 mL for liquids) are placed on a patch and applied to the test sites. They are secured with two layers of surgical gauze (1 in. [2.5 cm] squared) and tape. The animal is wrapped in cloth so that the

TABLE 44.1
Draize Scoring System

Erythema	
No erythema	0
Slight erythema	1
Well-defined erythema	2
Moderate or severe erythema	3
Severe erythema or slight eschar formation (injuries in depth)	4
Edema	
No edema	0
Very slight edema	1
Slight edema (well-defined edges)	2
Moderate edema (raised >1 mm)	3
Severe edema (raised >1 mm and extending beyond the area of exposure)	4

Source: Patrick, E. and Maibach, H. in *Current Topics in Contact Dermatitis*, Springer, New York, 1989.

TABLE 44.2
Modified Draize Irritation Method

Draize		FHSA	FIFRA	DOT	OECD
No. of animals	3	6	6	6	6
Abrasion	Yes	Yes	2 of each	No	No
Exposure period (h)	24	24	4	4	4
Examination (h)	24, 72	24, 72	0.5, 1, 24, 48, 72	4, 48	0.5, 1, 24, 48, 72
Excluded from testing	—	—	Toxic materials pH 2 or 11.5	—	Toxic materials pH 2 or 11.5

Note: FHSA, Federal Hazardous Substance Act; FIFRA, Federal Insecticide, Fungicide and Rodenticide Act; DOT, Department of Transportation; OECD, Organization for Economic Cooperation and Development.

Source: Bashir, S. and Maibach, H. in *Hand Eczema*, CRC Press, New York, 2000, 367–376.

patches are secure for a 24-h period. Assessment of erythema and edema, utilizing the scale noted in Table 44.1, takes place 24 and 72 h following patch application. Severe reactions are again assessed on days 7 or 14. Radiolabeled tracers or biochemical techniques to monitor skin healing is also utilized by some investigators. Other investigators supplement with histological evaluation of skin tissue (Mezei et al., 1966; Murphy et al., 1979).

The Draize test ultimately quantifies irritation with the primary irritation index (PII), which averages the erythema and edema scores of each test site and then adds the averages together. Materials producing a PII of <2 are considered nonirritating, 2–5 mildly irritating, >5 severely irritating and require precautionary labeling. Subsequent studies have demonstrated that the PII is somewhat subjective because the scoring of erythema and edema require clinical judgment (Patil et al., 1998).

Main critics of the Draize test oppose the harsh treatment of animals. They argue that the Draize test is unreliable in distinguishing between mild and moderate irritants. Furthermore, they believe the Draize is not an accurate predictor of skin irritancy as it does not include vesiculation, severe eschar formation, or ulceration in evaluating the PII. Finally, they argue that the Draize procedure is not reproducible (Weil and Scala, 1971) and they question its relevance with regard to human experience (Edwards, 1972; Nixon et al., 1975; Shillaker et al., 1989). Proponents of the Draize test point out that the test is somewhat inaccurate but it generally overpredicts the severity of skin damage produced by chemicals, and thereby errs on the side of safety for the consumer (Patil et al., 1996). This topic is hotly debated. In the meantime, the Draize assays are recommended by regulatory bodies.

44.2.2 Modified Draize Models

The Draize test has been modified in response to harsh criticisms over the past years. Alterations include changing the preferred species, use of fewer animals, testing on only intact skin and reduction of the exposure period to irritants. Please note Table 44.2 for a comparison of the modified Draize tests.

44.2.3 Cumulative Irritation Assays

Frequently, ICD is produced through cumulative exposure to a weak irritant. While the Draize assay assesses acute exposure to a strong irritant, there have been many assays developed to measure repetitive, cumulative irritation. One such assay was developed by Justice et al. (1961). They measured epidermal erosion through a repeat animal patch (RAP) test for comparing irritant potential of surfactants. In their study, solutions were occlusively applied to the clipped dorsum of albino mice for a 10-min interval. The process was repeated seven times and the skin was subsequently examined microscopically for epidermal erosion.

The repetitive irritation test (RIT), as described by Frosch et al. (1993), utilizes guinea pigs as the animal model in determining the protective efficacy of creams against various chemical irritants. In one study, the irritants sodium hydroxide (NaOH), sodium lauryl sulfate (SLS), and toluene were administered daily for 2 weeks to shaved dorsal skin of guinea pigs. Barrier creams were applied 2 h prior to and immediately following irritant exposure. Visual scoring, laser Doppler flowmetry (LDF), and transepidermal water loss (TEWL) quantified resultant erythema. The study found one barrier cream effective against SLS and toluene injury, while another barrier cream studied did not show any efficacy. In general, the RIT is most useful in evaluating the efficacy of barrier creams in preventing cumulative irritation.

To rank products for their irritant potential, repeat application patch tests have been developed. Diluted potential irritants are occlusively applied to the same site for 15–21 days. The sensitivity of the test is influenced by both the duration

of occlusion and the type of patch used to apply the irritants. In general, a longer occlusive period will result in enhanced percutaneous penetration. Similarly, the Draize-type gauze dressing will produce less percutaneous penetration as compared to the Duhring metal chambers. To facilitate interpretation of test results, a reference material that is of similar use or which produces a known effect is incorporated into the test. Rabbits and guinea pigs are the most commonly used animal species in the repeat application test (Phillips et al., 1972; Wahlberg, 1993). In a recent study, Kobayashi et al. (1999) studied the effects of propranolol as an irritant utilizing both primary and cumulative irritation assays. In both assays, skin irritation and histopathological changes were observed in all guinea pigs treated with propranolol, and those tended to increase with the increase of propranolol dosage. The skin reactions increased with the application times of propranolol up to 7 days in the cumulative skin irritation study. Scoring of the test sites were made in accordance with the following scale: 0 = no reaction, 1+ = mild erythema covering the entire patch area, 2+ = erythema and edema, 3+ = erythema, edema, and vesicles, 4+ = erythema, edema, and bullae.

One variation of the repeat application patch test involves measuring the edema-producing capacity of irritants utilizing a guinea pig model. Visual inspection and Harpenden calipers measure skin thickness following application of irritants for 3–21 days. This model demonstrates clear dose–response relationships and discriminating power for all irritants, excluding acids and alkalis (Wahlberg, 1993).

Open application assays, developed by Marzulli and Maibach (1975), involve application of irritants onto the backs of rabbits 16 times over a 3-week period. Visual scoring of erythema and skin thickness measurements are utilized to quantify results. A high degree of correlation has been observed when comparing erythema and skin thickness data. In addition, the results of 60 test substances in rabbits strongly correlated with the results of cumulative irritation studies in man, suggesting that the rabbit assay is a useful model.

A modified open application assay was performed by Anderson et al. (1986). In his assay, irritants are applied once a day for 3 days to a 1-cm^2 test site on the backs of guinea pigs. Sites are evaluated visually for erythema and edema. In addition, biopsies are taken and skin samples are stained with May–Grunward–Giemsa under oil immersion, to evaluate epidermal thickness and dermal infiltration. Irritants are compared with the standard irritant, 2% SLS, and their potency is ranked. Extensive processing involved in properly performing this assay may limit its usefulness.

44.2.4 Immersion Assay

Aqueous detergent solutions and other surfactant-based products are evaluated for irritancy using the guinea pig immersion assay (Calandra, 1971; MacMillan et al., 1975; Gupta et al., 1992). This assay involves placing 10 guinea pigs in a restraining device that is immersed in a 40°C test solution for 4 h daily for a total of 3 days (Kooyman and Snyder, 1942). The restraining apparatus allows the guinea pig's head to be above the solution. Twenty-four hours following the final immersion, the animals' flanks are shaved and evaluated for erythema, edema, and fissures. In one study, the dermatotoxic effects of detergents in guinea pigs and humans were concomitantly tested (Gupta et al., 1992). The immersion assay was utilized to test guinea pigs, while the patch assay tested humans. Irritation of guinea pig skin led to epidermal erosion and a 40–60% increase in histamine content. Seven of eight human subjects had a positive patch test to the same irritants, indicating a strong correlation between the guinea pig and human models.

44.2.5 Mouse Ear Model

The mouse ear model is used to evaluate the degree of inflammation associated with shampoos or surfactant-based products. Uttley and Van Abbe (1973) first described the mouse model when they applied undiluted shampoos to one ear of mice daily for 4 days. They visually assessed the erythema, vessel dilation, and edema. However, the anesthetic used to anesthetize the mice in this study may have altered the development of inflammation and confounded results.

More recently, Patrick and Maibach (1987) applied surfactants to measure mouse ear thickness at various time points following irritant application. Pretreating the ear with croton oil or 12-*O*-tetradecanoylphorbol 13-acetate 72 h prior to irritant application increased the sensitivity of the assay. This assay was most useful in testing surfactant-based products and had little efficacy with oily or highly perfumed materials.

44.2.6 Recent Assays

Recent animal assays have been developed to quantify irritant response. Humphrey et al. (1993) measured Evans blue dye recovered from rat skin after exposing the skin to inflammatory agents. Trush et al. (1994) assessed the dermal inflammatory response to numerous irritants by measuring the level of myeloperoxidase enzyme in polymorphonuclear leukocytes in young CD-1 mice.

44.2.7 Conclusion

Animal assays must be interpreted with caution. Dose–response measurements must be followed. Draize scores are most accurate when compared to related compounds with a record of human exposure. It is important to note that occlusive application does not enhance percutaneous penetration for all materials.

Responses in animal models, particularly the guinea pig and the rabbit, have a high degree of correlation to those of humans, but some inconsistencies have occurred. Major discrepancies in irritant response between different animal species tested under identical conditions have occurred (Llewellyn et al., 1972; Gilman et al., 1978), particularly with regard to weak irritants and colored materials. Subjective visual scoring techniques have accounted for some of these discrepancies. It is prudent to utilize other methodologies in addition to the animal model when evaluating a putative irritant.

44.3 *IN VITRO* ASSAYS

In vitro skin irritation assays are of potential benefit in addressing humane concerns associated with animal testing. These "alternative" methods may potentially reduce the number of animals needed in irritation testing, or in some cases may fully replace the need to use animals. In recent years, a number of *in vitro* skin irritation assays have been developed. However, most of these have not been evaluated in validation studies to determine their usefulness, limitations, and compliance with regulatory testing requirements. Furthermore, dose–response relationships have not been established for *in vitro* methods.

Studies evaluating *in vitro* testing thus far indicate usefulness in predicting starting doses for *in vivo* studies, potentially reducing the number of animals used for such determinations. Additionally, other studies suggest an association between *in vitro* cytotoxicity and human lethal blood concentrations. The U.S. Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) and the U.S. National Toxicology Program Center for the Evaluation of Alternative Toxicological Methods (NICEATM) were established to evaluate *in vitro* irritant testing. To date, there are four approved irritation assays, namely, Corrositex®, EpiDerm™, EPISKIN™, and Rat Skin Transcutaneous Electrical Resistance (TER) Assays.

Corrositex is a collagen matrix acting as synthetic skin, and is used to assess the dermal corrosivity potential of chemicals. Should a chemical pass through the biobarrier by diffusion or destruction, Corrositex elicits a color change in the underlying liquid chemical detection system (CDS). Corrositex is currently used by the U.S. Department of Transportation (U.S. DOT) to assign categories of corrosivity for labeling purposes according to United Nations (UN) guidelines. However, its use is limited to specific chemical classes, including acids, acid derivatives, acylhalides, alkylamines and polyalkyamines, bases, cholorosilanes, metal halides, and oxyhalides.

A peer review panel of NICEATM and ICCVAM elucidated some of the advantages to Corrositex, including its possible usefulness in replacing or reducing the number of animals required. Positive test results often eliminate the need for animal testing. When further animal testing is necessary, often only one animal is required to confirm a corrosive chemical. The panel also concluded that most of the chemicals identified as negative by Corrositex or nonqualifying in the detection system are unlikely to be corrosive when tested on animals for irritation potential.

EpiDerm(EPI-200) is a three-dimensional human skin model that uses cell viability as a measure of corrosivity. It has been utilized with several common tests of cytotoxicity and irritancy, including MTT, IL-la, PGE_2, LDH, and sodium fluorescein permeability.

EPISKIN is a three-dimensional human skin model comprised of a reconstructed epidermis and a functional stratum corneum. In a study supported by the European Center for the Validation of Alternative Methods (ECVAM), EPISKIN was useful in testing all types of potential irritants, including organic acids, organic bases, neutral organics, inorganic acids, inorganic bases, inorganic salts, electrophiles, phenols and soaps/surfactants. With both EPISKIN and EpiDerm, the test material is topically applied to the skin for up to 4 h with subsequent assessment of the effects on cell viability.

***In Vitro* Assays**

Assay	Description	Methodology
Corrositex	Collagen matrix acting as synthetic skin	A color change in the underlying liquid chemical detection system when irritant passes through matrix
EpiDerm	Three-dimensional matrix acting as synthetic skin	Cell viability as a measure of corrosivity
EPISKIN	Three-dimensional matrix acting as synthetic skin	Cell viability as a measure of corrosivity
TER	Skin disks taken from the pelts of humanely killed young rats	Significantly lower inherent transcutaneous electrical resistance when skin barrier is compromised

In the TER Assay, irritants will portray a loss of normal stratum corneum integrity and barrier function. A reduced barrier function will exhibit a significantly lower inherent transcutaneous electrical resistance. TER involves up to 24 h application of test material to the epidermal surfaces of skin disks taken from the pelts of humanely killed young rats. Comparing EpiDerm, EPISKIN, and TER, only EPISKIN was able to significantly distinguish between two particular types of chemicals. Currently, the ICCVAM recommends that EpiDerm, EPISKIN, and TER are used to assess the dermal corrosivity potential of chemicals in a "weight-of-evidence" approach. In general, positive corrosivity tests will not require further testing, while negative corrosivity will.

In vitro assays are promising and have significant interest to toxicologists. The future promises a greater use for *in vitro* irritancy testing.

44.4 HUMAN MODELS

Following the development of the patch test, Draize et al. suggested a 24-h single-application patch test in humans. Human testing facilitates extrapolation of data to the clinical setting. Many variations of the single-application test have been developed. Testing is often performed on undiseased skin (Skog, 1960) of the dorsal upper arm or back. The required test area is small and up to 10 materials may be tested simultaneously and compared. A reference irritant substance is often included to account for variability in test responses. In general, screening of new materials involves open application on the back or dorsal upper arm for a short amount of time (30 min to 1 h) to minimize potential adverse events in subjects.

44.4.1 Single-Application Patch Testing

The National Academy of Sciences (National Academy of Sciences and Committee for the Revision of NAS Publication 1138, 1977) recommended a 4-h single-application patch test protocol for routine testing of skin irritation in humans. In general, patches are occluded onto the dorsal upper arm or back skin of patients. The degree of occlusion varies according to the type of occlusive device; the Hilltop or Duhring chambers or an occlusive tape will enhance percutaneous penetration as compared to a nonocclusive tape or cotton bandage (Patil et al., 1996). Potentially volatile materials should always be tested with a nonocclusive tape.

Exposure time to the putative irritant varies greatly, and is often customized by the investigator. Volatile chemicals are generally applied for 30 min to 1 h while some chemicals have been applied for more than 24 h.

Following patch removal, skin is rinsed with water to remove residue. Skin responses are evaluated 30 min to 1 h following patch removal to allow hydration and pressure effects of the patch to subside. Another evaluation is performed 24 h following patch removal. The animal Draize scale is used to analyze test results (see Table 44.1). The Draize scale does not include papular, vesicular, or bullous responses; other scales have been developed to address these needs.

Single-application patch tests generally heal within 1 week. Depigmentation at the test site results in some subjects.

44.4.2 Cumulative Irritation Test

Utilizing statistical analysis of test data, Kligman and Wooding (1967) calculated the IT50 (time to produce irritation in 50% of subjects) and ID50 (dose required to produce irritation in 50% of subjects following a 24-h exposure). Their work formed the basis for the 21-day cumulative irritation assay. The "21 day assay" is used to screen new formulas prior to marketing. The original assay involved application of an 1-in. (2.5 cm) square of Webril saturated with the test material (either liquid or 0.5 g of viscous substance) to the skin of the undamaged upper back. Occlusive tape secured the patch. Twenty-four hours after patch application, the test site is examined and the patch is reapplied. The test is repeated for 21 days.

Two modifications of the cumulative irritation test were studied by Wigger-Alberti et al. (1997a). One assay involved Finn chamber application of metal-working fluids onto the midback of volunteers for 1 day. The sites were evaluated and the fluids were then reapplied for an additional 2 days. In the other assay, a 2-week, 6-h/day repetitive irritation test (excluding weekends) was utilized. Better discrimination of irritancy and shorter duration was observed with the 3-day model.

44.4.3 Chamber Scarification Test

The chamber scarification test assesses the irritancy potential of materials on damaged skin (Frosch and Kligman, 1976; 1977). Subjects included in this assay are highly sensitive to 24 h exposure to 5% SLS (they form vesicles, severe erythema, and edema postapplication). Six to eight 10 mm^2 areas on the volar forearms are scratched eight times with a 30-gage needle. Scarification damages the epidermal layer without drawing blood. Four scratches are parallel and the other four are perpendicular to the test site. 0.1 g of test material (or 0.1 mL of liquid) is then applied to the scarified area for 24 h via Duhring chambers. Nonocclusive tape is used to secure the chambers in place. With fresh specimens, patches are applied daily for 3 days. A visual scoring scale is used to quantify test results 30 min following patch removal. An analogous area of intact skin must be scored as well, so that evaluation is based upon comparison between compromised and intact skin. The visual score of scarified test sites divided by the score of intact test sites, known as the scaarification index, allows this comparison to be made. The relationship of this assay to prediction of irritant response from routine use has yet to be established.

44.4.4 Immersion Tests

Patch tests often overpredict the irritant potential of some materials. Immersion tests were established to improve irritancy prediction by mimicking consumer use. Kooyman and Snyder (1942) developed the arm immersion technique to compare the relative irritancy of two soap or detergent products. Soap solutions of up to 3% are prepared in troughs and subjects immersed one hand and forearm in each trough, comparing different products or concentrations. Temperature is maintained at 41°C (105°F). The exposure period varies between 10 and 15 min a day for a total of 5 days or until observable irritation is produced on both arms. The antecubital fossa is generally the first area to experience irritation, followed by the hands (Justice et al., 1961; Kooyman and Snyder, 1942).

More recently, variations on the arm immersion technique have developed so that the antecubital fossa and the hands are separately tested. Variations incorporate different dosing regimens or measuring different endpoints. Clarys et al. (1997) and Clarys and Barel (1997) investigated the effects of temperature and anionic character on the degree of irritation caused by detergents. TEWL, erythema (colorimetry, a* parameter), and skin dryness (capacitance) were used to quantify test results. The irritant response was increased by higher temperature and higher anionic content. Utilizing a modified arm immersion technique, Allenby et al. (1993) noticed that once skin had been compromised (erythema of 1+ on a visual scale), irritants applied to the forearm and back caused an exaggerated response.

44.4.5 Soap Chamber Technique

The "chapping" potential of bar soaps is evaluated with the soap chamber technique, developed by Frosch and Kligman (1979). While patch testing is useful in predicting erythema, it does not address the dryness, flaking, and fissuring observed with bar soap use. Using this method, 0.1 mL of an 8% soap solution is applied to the forearm via Duhring chambers fitted

with Webril pads. Nonocclusive tape is used to secure the chambers. Patches are applied for 24 h on day 1 and 6 h on days 2–5. If severe erythema at the test site occurs, the investigator must discontinue the study. Skin responses are evaluated with visual scoring of erythema, scaling, and fissures. This test correlates well with skin-washing procedures but tends to overpredict irritant response of some materials.

44.4.6 Protective Barrier Assessment

The skin barrier function assays test the efficacy of protective creams in preventing an irritant response. Zhai et al. (1998) studied the effect of barrier creams in reducing erythema, edema, vesiculation, and maceration. Subjects were given creams and then irritated with either SLS or ammonium hydroxide. Paraffin wax in cetyl alcohol was the most effective in preventing irritation. In another study by Wigger-Alberti and Elsner (1997a), petrolatum was applied to the backs of 20 subjects. Subjects were then exposed to SLS, NaOH, toluene, and lactic acid. Irritation was assessed by visual scoring, TEWL, and colorimetry. Petrolatum was found to be an effective barrier cream against SLS, NaOH, and lactic acid and moderately effective against toluene.

Frosch et al. (1993) revised the RIT (see Section 44.2) to evaluate the effect of two barrier creams in preventing SLS-induced irritation. The irritant was applied to the ventral forearms of human subjects for 30 min daily for 2 weeks. Visual scoring, LDF, colorimetry, and TEWL were utilized to assess resultant erythema. TEWL was found most useful in quantifying results, while colorimetry was the least beneficial.

44.4.7 Bioengineering Methods

Modern bioengineering methods utilized to quantify test results include TEWL, capacitance, ultrasound, LDF, spectroscopy, and chromametry (colorimetry). Most of the assays described were developed before the introduction of these bioengineering methods. These methods allow a more precise quantification of test results. These techniques are described in detail by Patil et al. (1998).

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45 Kawai Method for Testing Irritation

Keiichi Kawai

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45.1 INTRODUCTION

Evaluation of skin irritancy is important because it may be useful in the prevention of irritant contact dermatitis. Irritant contact dermatitis is a multifactorial disease and it embraces a wide spectrum of reactions where different mechanisms are involved. Therefore, there is no single method to evaluate and predict all kinds of irritant contact dermatitis.

When irritancy is strong, visible inflammatory changes (acute irritant contact dermatitis) occur on the skin, and the evaluation of strong irritancy is relatively easy. When irritancy is weak, no visible reaction is induced. However, repeated and damaging insults to the skin by weak irritants often cause skin barrier dysfunction and cumulative irritant dermatitis. It is difficult to predict such weak irritation to the skin. Using recent bioengineering techniques such as evaporimeter, laser-Doppler flowmetry, ultrasound, impedance meter, colorimeter, etc., several investigators have tried to evaluate nonvisible cutaneous changes due to weak skin irritancy. As these methods apparently give information about different aspects in cutaneous irritation and skin function,[1] weak skin irritancy cannot be fully evaluated by any single method.

Kawai's method/nitrocellulose replica method is used to predict weak skin irritancy. To observe such a subtle skin damage, Kawai[2] observed replicas of stratum corneum microscopically and reported several skin surface changes occurring after irritant application in spite of no visible inflammatory changes. Using the replica method, we have evaluated over 18,000 commercial products including new chemicals, cosmetics, textiles, etc., and discovered that the incidence of complaints for skin irritation against tested products correlated well with our evaluations for skin safety.[3] In this chapter, we address Kawai's method/nitrocellulose replica method concisely.

45.2 METHOD

45.2.1 Subjects

A panel for a test consists of 20 healthy adults with no less than one-third belonging to either sex. They have no past or present history of skin disease. Informed consent is obtained from all subjects in each test. About 140 people are registered for the test and people who have participated in a test performed within the past month are not eligible for another test.

45.2.2 Test Samples and Control Substance

The test is applied to substances to be used in contact with the skin such as textiles, cosmetics, detergents, drugs, etc. As the aim for the Kawai method is to evaluate weak irritancy, as is the case with daily commodities, final products rather than raw materials are usually tested. For the safety of the human volunteer, unknown chemicals and known corrosive and severe irritants are not applied to the test.

Susceptibility to irritants differs by individual. Thus, there may be differences of susceptibility among each panel and the irritancy measured using one panel may be different from the irritancy measured using another panel. To avoid this, we use the same control substance in all tests. Every time we test, the reaction of the control substance is observed. The control substance and its preparation method are as shown in Table 45.1.

45.2.3 Irritant Application

Test substances are applied on the inner sides of the upper arm by semi-open test (Figure 45.1): they are applied directly onto a 1.5 cm^2 area of skin, and then two pieces of 2 cm^2 gauze are put on each sample and fixed gently with paper plaster. Thus, the substances are not completely occluded as closed patch tests and they approach a natural condition as found in daily life (for example, clothing, cosmetics, medicaments, etc.). The patches are removed after 24 h and the skin replicas are taken 30 min later.

Recently, we showed that occlusive patch tests can cause significant skin surface changes by observing the replicas microscopically.[4] Lindberg and Forslind[5] reported that simple occlusion for 3, 6, 24, and 48 h with aluminum cups used for patch testing causes morphological alterations in Langerhans cells, apposition of mononuclear cells, and increased number of mononuclear cells in the epidermis. Kligman[6] also mentioned that occlusion of the skin by polyethylene films can induce cytologic damage to the epidermis and dermis in as little as 48 h. Thus, closed patch tests are not appropriate for the replica method. We confirmed that the semi-open test for 24 h did not cause morphological changes on the skin surface.

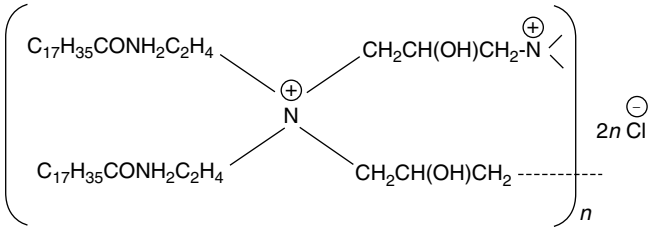
45.2.4 Obtaining the Skin Replica

The procedure of taking skin replicas is shown in Figure 45.2. Specifically, a nitrocellulose disk, which is a round and translucent plate 21 mm in diameter, is coated evenly with *n*-butyl acetate on the site of the patch tests, so that the disk surface becomes softened. Then, the disk is applied carefully to the skin surface. After 1–2 min, when the disk surface is solidified, the disk is removed gently from the skin surface and is attached onto a mount.

TABLE 45.1
Control Substance—Di(Stearo Amino Ethylene)Amide Epichlor Hydrine Condensate

Ingredients

Octadecanamid *N N′* -(imino diethylene) bis, polymer with 1-chloro-2,3-epoxypropane



Preparation

Cloth used: Gauze of Japanese pharmacopia (washed by rubbing in warm water and dried)
Concentration of treatment: 2.5% aqueous solution

Soaking: 1 dip 1 nip (at 40°C for 20 s)
Wet pick up: 100%

Drying: 100°C for 7 min
Target add-on: 1% (to the pure fibrous content)

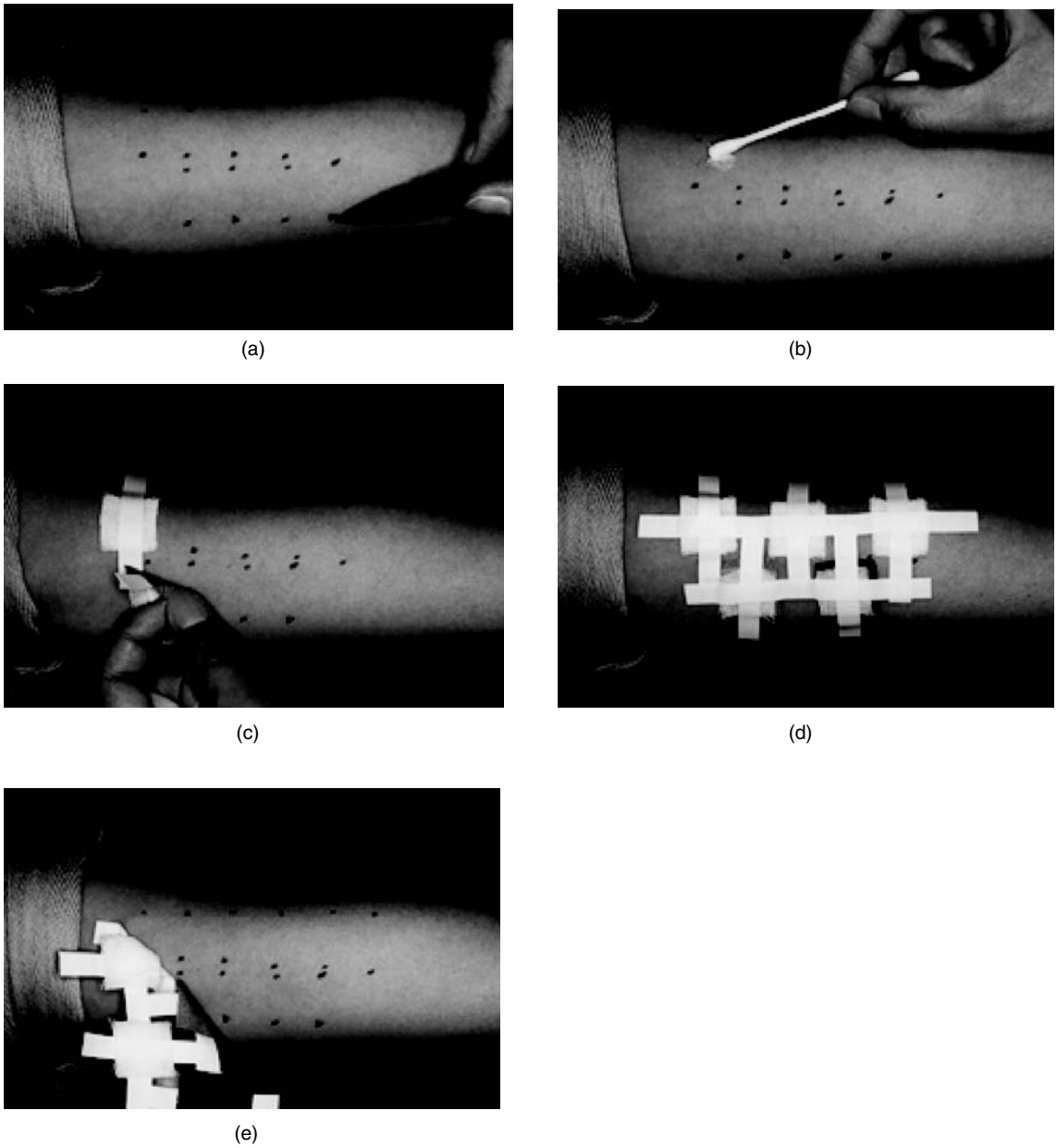


FIGURE 45.1 Irritant application. (a) Indicate the test sites with a marker. (b) A 1.5 cm^2 patch is applied per test sample. (c) Two pieces of gauze, 2 cm^2, are put on each sample and secured by paper plaster. (d) One test consists of four points each on the right and left forearms: eight patches in total of which one is a control sample. (e) After 24 h of application, the patches are removed. The test sites for this method are *the inner sides of the upper arm*.

45.2.5 Observation

The specimens are observed from all fields carefully under a microscope with a 50× magnification. We use a binocular microscope and a stereomicroscope.

45.3 OBSERVED REPLICA PICTURES UNDER MICROSCOPE

A replica finding of the normal skin of the inner side of the arm is shown in Figure 45.3. Several skin surface changes are observed after an irritant patch testing, including round ridges (Figure 45.4), the appearance of deepened skin furrows (Figure 45.5), membranous desquamation (Figure 45.6, a phenomenon in which the corneum is detached as a thick membrane), and loss of normal triangular configuration of the skin furrow pattern (Figure 45.7).

45.3.1 Normal Skin

In normal cases, the skin replica of the inner sides of the upper arm shows a triangular configuration of skin furrows

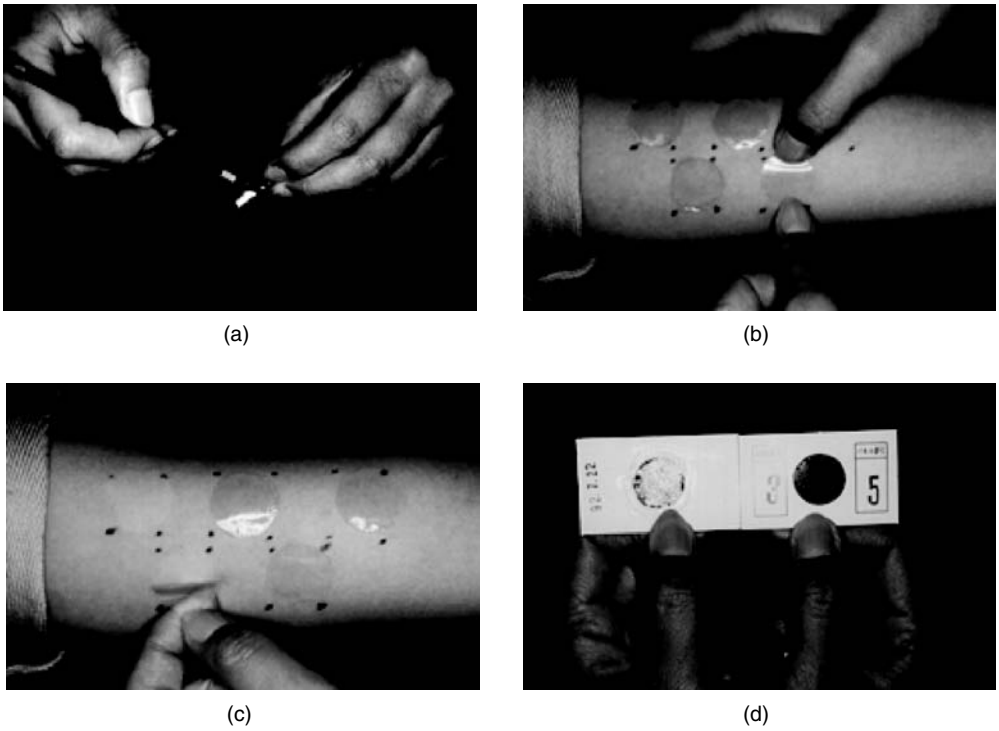


FIGURE 45.2 Obtaining the skin replica. (a) A nitrocellulose disk is coated evenly with *n*-butyl acetate. (b) The disk is applied carefully to the skin surface. (c) After 1–2 min, the disk is removed gently from the skin surface. (d) The resulting skin replica is attached to a mount with a hollow center. (Note that care should be taken not to touch the replica surface.)

seen as a thin line at low magnification (×10–×50). Under stronger magnification (×100), the tertiary lines, which are the limits of the corneocytes, can be seen.

Structural relief of skin replicas from various body sites is not constant. We believe that the inner side of the upper arm is the best site for irritant application, because structural relief of the replicas and the resulting data from the inner sides of the upper arm is most constant and reproducible.[7]

45.3.2 Round Ridges

Round ridges are a phenomenon in which round or curved lines are observed in the triangular ridges (Figure 45.4). It is considered that this change is due to transient, microscopic edema of the horny layer of the skin. Round ridges are seen in skin, which has been wiped with diluted soap solution or alcohol, and can also be seen in about 40% of generally healthy individuals.

45.3.3 Deepened Skin Furrows

The appearance of deepened skin furrows is the most frequent and sensitive change which occurs on the skin after irritant application. This change is well observed under a stereomicroscope. As shown in Figure 45.5, normal skin shows thin lines running along skin furrows, whereas an abnormal region shows wide translucent bands, which indicates deepened skin furrows.

Although skin with the change of deepened skin furrows develops no visible inflammatory changes such as erythema and edema, it shows intercellular edema in the epidermis and exocytosis of mononuclear cells microscopically (Figure 45.8). This means epidermal inflammatory changes occur before the damage becomes clinically evident. Interestingly, there is no significant change in the horny layer.

45.3.4 Membranous Scale

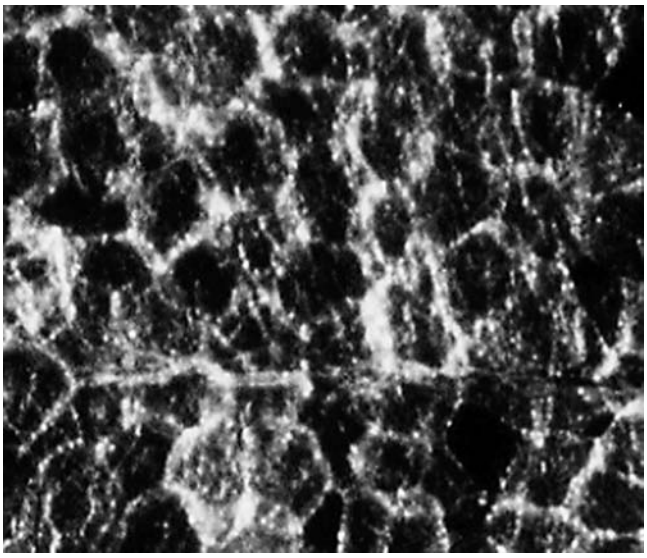
This is a phenomenon in which the corneum is detached as a thick membrane (Figures 45.6 and 45.9). This change is thought to be the result of severe damage of the stratum corneum. It is observed after strong irritant application or after adhesive tape is removed from the skin.

45.3.5 The Loss of the Triangle Configuration of the Skin Furrow Pattern

The loss of the triangle configuration of the skin furrow pattern is caused by two different mechanisms (Figure 45.9): one is the result of destructive changes of the skin furrows by severe damage of the stratum corneum. Membranous desquamation is frequently observed with this destructive change. Thus it may be observed when the replica is taken after the removal of



(×50 under a microscope)



(×100 under a stereomicroscope)

FIGURE 45.3 Normal replica picture of the skin. In normal cases, the skin replica shows triangular configuration of skin furrows seen as thin lines. Under higher magnification (×100), the tertiary lines, which are the limits of the corneocytes, can be seen.

membranous scale. It may also be the result of severe dermal edema. Contact urticaria to balsam of Peru caused the loss of the skin furrow pattern.[8] As the skin furrows are pushed up by dermal edema, they become shallow or disappear. The change by the urticaria, however, does not cause the loss of the normal triangular pattern, and it occurs only after severe urticaria. Probably loss of triangular minor furrows by the destructive change of the horny layer is much more frequently observed in irritant patch testings than those by dermal edema.

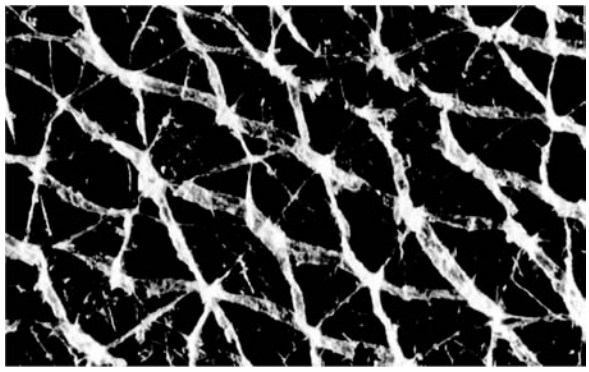
45.3.6 Replica Pictures and the Degree of Skin Irritancy

Figure 45.10 summarizes the relationship between replica pictures and the degree of skin irritancy. Round ridges and deepened skin furrows are thought to be reversible changes



(×50 under a microscope)

FIGURE 45.4 Round ridges. Round ridges are a phenomenon in which round or curved lines are observed in the triangular ridges. It is considered that this change is due to transient, microscopic edema of the horny layer of the skin. Round ridges are seen in the skin, which has been wiped with diluted soap solution or alcohol, and can also be seen in about 40% of generally healthy individuals.



(×50 under stereomicroscope)

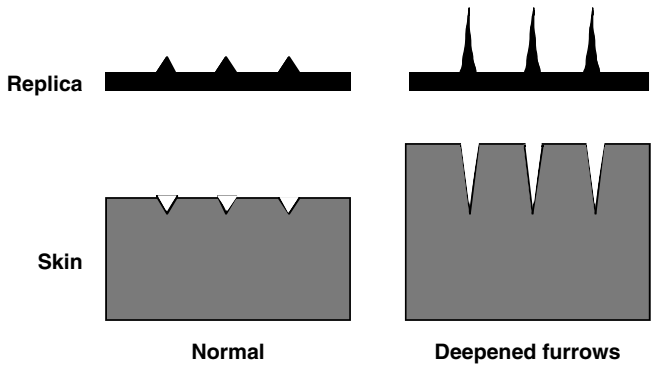
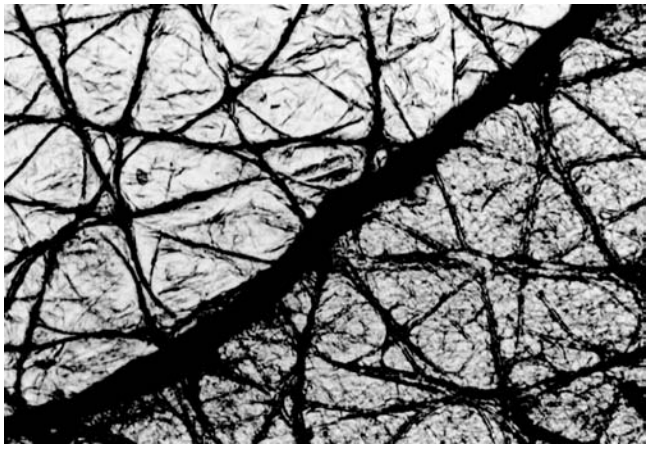


FIGURE 45.5 Deepened skin furrows. When observed under a stereomicroscope, normal skin shows thin lines running along skin furrows, whereas the abnormal region shows wide translucent bands, which indicate deepened skin furrows. This is the most sensitive and frequent change.



(×50 under a microscope)

FIGURE 45.6 Membranous desquamation. A phenomenon in which the corneum is detached as a thick membrane. This occurs only after strong irritant application. Stratum corneum are morphologically changed to the scaly skin.

and the results of mild damage of the skin, while membranous desquamation and loss of normal triangular configuration of the skin furrow pattern are thought to be irreversible changes and the results of more severe damage.

45.4 EVALUATION OF REPLICA PICTURES

45.4.1 Criteria for Judgment of Skin Irritancy

We have evaluated skin irritancy of products, which come into contact with the skin by a specific criterion for more than 30 years.

The replicas taken from the 20 subjects, after a 24-h semiopen patch test, are observed microscopically and the abnormal changes, including round ridges, deepened skin furrows, membranous desquamation, and loss of triangular configuration of the furrow, are recorded. According to the degree of abnormal reaction as observed in the replica, there are four ranks of judgment of skin irritancy. Criteria for this are as follows.

45.4.1.1 Negative

No change is observed both microscopically and macroscopically, or the abnormal reaction is less severe than the reaction to the control substance. As 40% of the normal skin shows round ridges, this change is not thought to be irritant reaction.

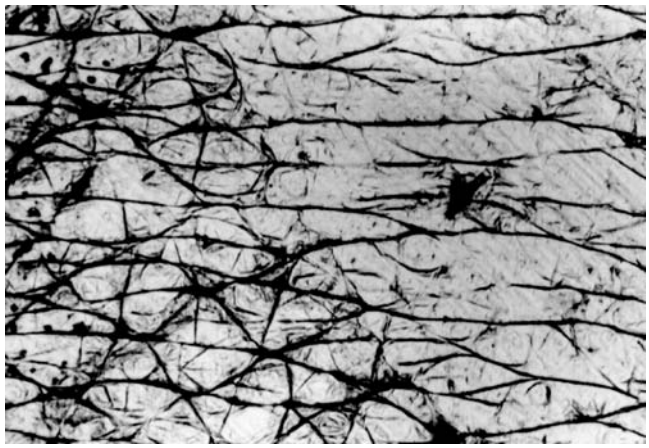
45.4.1.2 Grade 1 (Almost Negative)

B score is defined as the number of subjects who show deepened skin furrows from the control substance, subtracted from the number of subjects who show the same reaction from the test substance. In this case, B score is counted only when the replica shows deepened skin furrows for more than 50% of the area of the sample.

If the B score is <2, and there are no other changes such as loss of the triangle configuration of the skin furrows, membranous desquamation on the replica, or visible changes on the skin, the judgment is "grade 1 (almost negative)."



(×50 under a microscope)



(×50 under a microscope)

FIGURE 45.7 The loss of the triangle configuration of the skin furrow pattern. In Figure 45.7, the normal structure of the skin surface was destroyed and the triangular skin furrow pattern was lost. This is caused by two different mechanisms: one is the result of destructive changes of the skin furrows by severe damage of the stratum corneum. Membranous desquamation is frequently observed with this destructive change. Thus, this may be observed when the replica is taken after the removal of membranous scale. The other is the result of dermal edema. Actually, urticaria shows similar changes on the replica. As the skin furrows are pushed up by dermal edema, they become shallow or disappear. The change by urticaria, however, does not cause the loss of the normal triangular pattern. The loss of triangular minor furrows by the destructive change of the horny layer is much more frequently observed than those by dermal edema in irritant patch testings.

45.4.1.3 Grade 2 (Almost Positive)

When the B score is 3, and there are no other changes such as loss of the triangle configuration of the skin furrows, membranous desquamation on the replica, or visible change on the skin, the judgment is "grade 2 (almost positive)."

45.4.1.4 Grade 3 (Positive)

When the B score is >4, or if one or more subjects show loss of the triangle configuration of the skin furrow pattern or membranous desquamation, the judgment is "grade 3 (positive)."

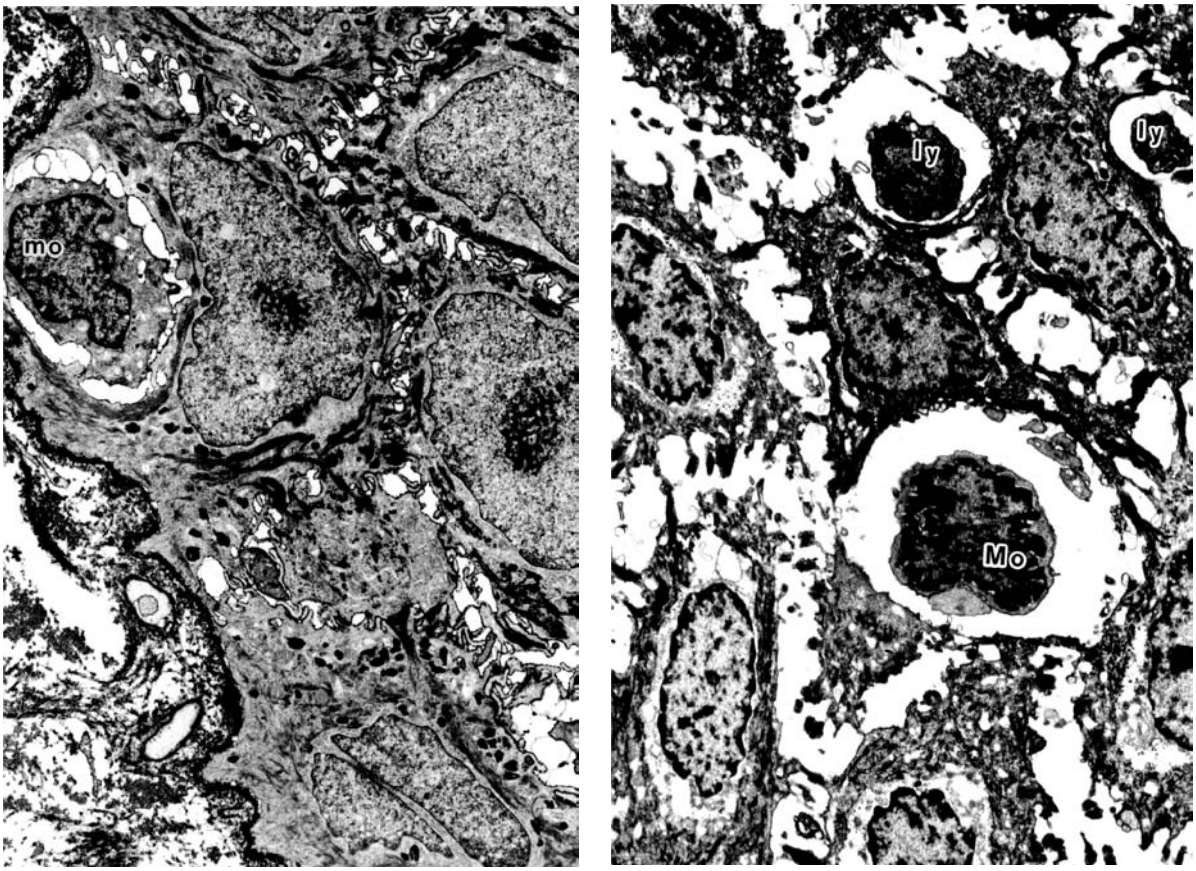


FIGURE 45.8 Histologic finding of the normal skin and the skin with deepened furrows. Epidermal intercellular edema and infiltration of mononuclear cells were found in the skin with deepened furrows.

45.4.1.5 Grade 4 (Positive Macroscopically)

When the skin shows any macroscopic inflammatory change (redness, swelling, etc.), the judgment is "positive macroscopically."

The judgment of "negative" and "grade 1 (almost negative)" means the test product has relatively weak subclinical irritancy, "grade 2 (almost positive)," and "grade 3 (positive)" mean it has relatively strong subclinical irritancy. Grade 4 means that the test product has clinically overt irritancy.

45.4.2 Follow-Up Study

Using the Kawai method, in the past 30 years we have evaluated more than 18,000 commercial products based on the criteria mentioned above. We have reviewed the incidence of complaints of skin irritation against tested products three times and the results were similar each time. From the results[3] of the third follow-up study, which investigated 2796 products tested from 1980 to 1991, the incidence of claims against products judged negative or grade 1 was 4.2%, and the incidence of claims against products judged grade 2 or grade 3 was 12.1%. Therefore, our judgments correlated well with the incidence of claims. We believe that the replica method can be used as a product test for screening skin irritancy.

45.4.3 Scoring of Deepened Skin Furrows and Experimental Investigations of Weak Skin Irritancy

The appearance of deepened skin furrows is the most frequent and sensitive change which occurs on the skin after irritant application. Thus, evaluation of this change is most important to judge the irritancy of products. The degree of appearance of the change was not considered in the criteria mentioned above. Only the number of subjects whose replicas show deepened skin furrows for more than 50% of the area of the sample is counted. Changes below 50% of the area are neglected. To adjust this neglection, reactions of deepened skin furrows are scored as follows (Figure 45.11): no skin furrow change scores 0, skin furrows which were deepened up to 25% of the area of the sample scores 0.5, from 25 to 50% scores 1, for more than 50% scores 2.

We have used the scoring system for several experimental investigations of skin irritancy. Studying the dose–response relationship for Sodium Lauryl Sulfate (SLS) in humans, the scoring of deepened skin furrows was a much more sensitive method than the visual scoring system using patch testings.[7] Similarly, several weak irritants for cosmetic materials such as liquid oil,[9] carboxylic acids, alcohols,

esters, and aldehyde with different chain length[10] have been evaluated. These studies showed that although no macroscopic alterations were found on the tested skin sites by closed patch testing, subclinical irritation could be evaluated by the scoring of deepened skin furrows. Recently, we demonstrated by using the scoring system of deepened skin furrows that subclinical irritation from liquid oil used as cosmetic ingredients is highly correlated with a reciprocal of molecular radius.[10]

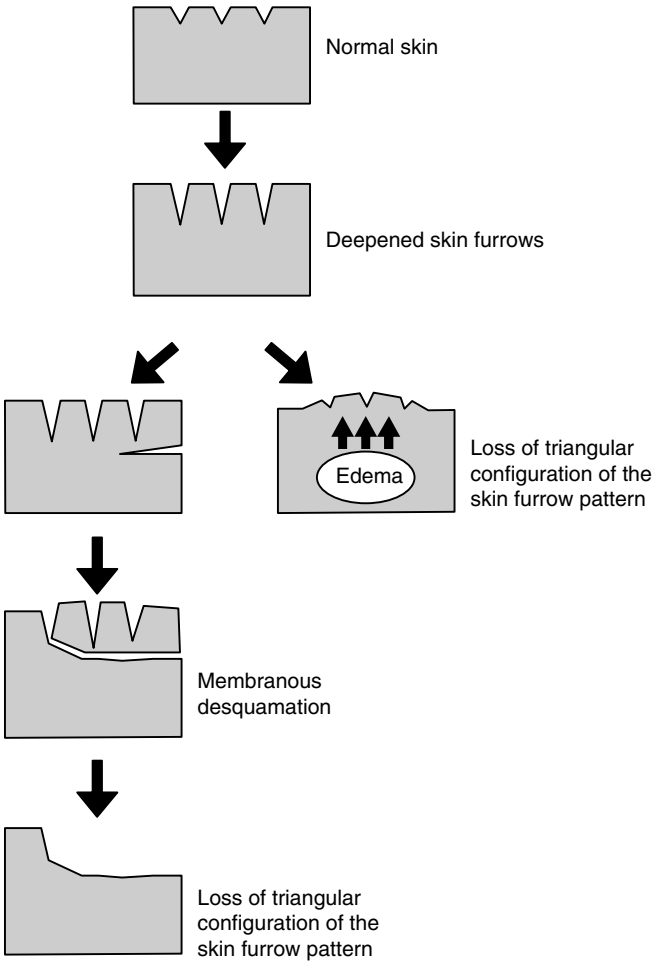


FIGURE 45.9 Possible mechanism of skin surface changes after irritant applications.

Not only chemical irritation but also physical irritation can be evaluated by the Kawai method. By using the Kawai method, Kondo et al.[11] reported that the physical skin irritation was dependent on fiber cross-sectional shape, fiber thickness, and yarn twist. Moreover, they have shown that the measurement of fabric mechanical properties and handles of hard-finished fabrics were closely related with their skin irritation (the degree of hardness of the fabric correlated to skin irritancy).[12] Tokumura et al.[13] showed that the cumulative amount of stripped corneocytes by repetitive application of adhesive tape correlated well with the appearance of deepened skin furrows on the tested skin.

45.5 SUMMARY

The Kawai method is discussed in this chapter. The Kawai method is used to predict weak skin irritancy. Using this method, we have evaluated the effects of commercial products on the skin, and our judgments based on the changes of replicas correlated well with the incidence of claims for skin troubles. Thus, we believe that the replica method can be used as a product test for screening skin irritancy. However, any predictive method, including this method, cannot be a precise method to assess skin irritancy, because the development of skin irritancy depends on various factors such as concentration or exposure time, chemical or physical property, cumulative effect with other irritants, environmental conditions, individual susceptibility, and so forth. As the replica method is merely evaluating skin surface changes after weak irritancy, this method can only evaluate the irritants which mainly affect the horny layer. Thus, several different methods must be combined for more precise prediction of skin irritancy.

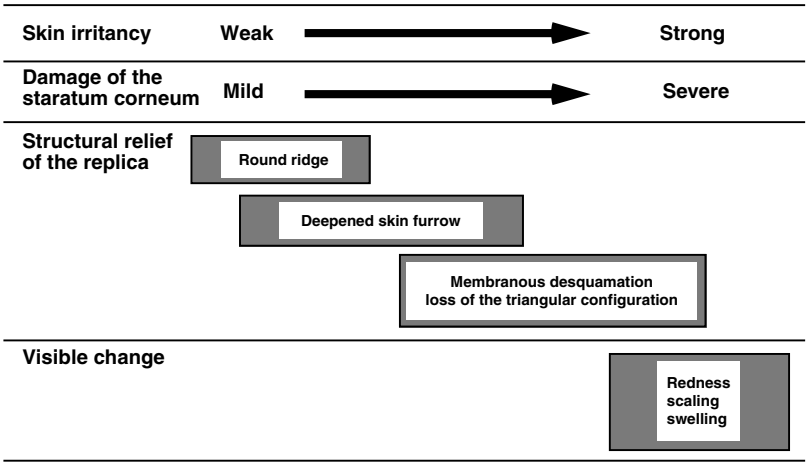


FIGURE 45.10 Replica pictures and the degree of skin irritancy.

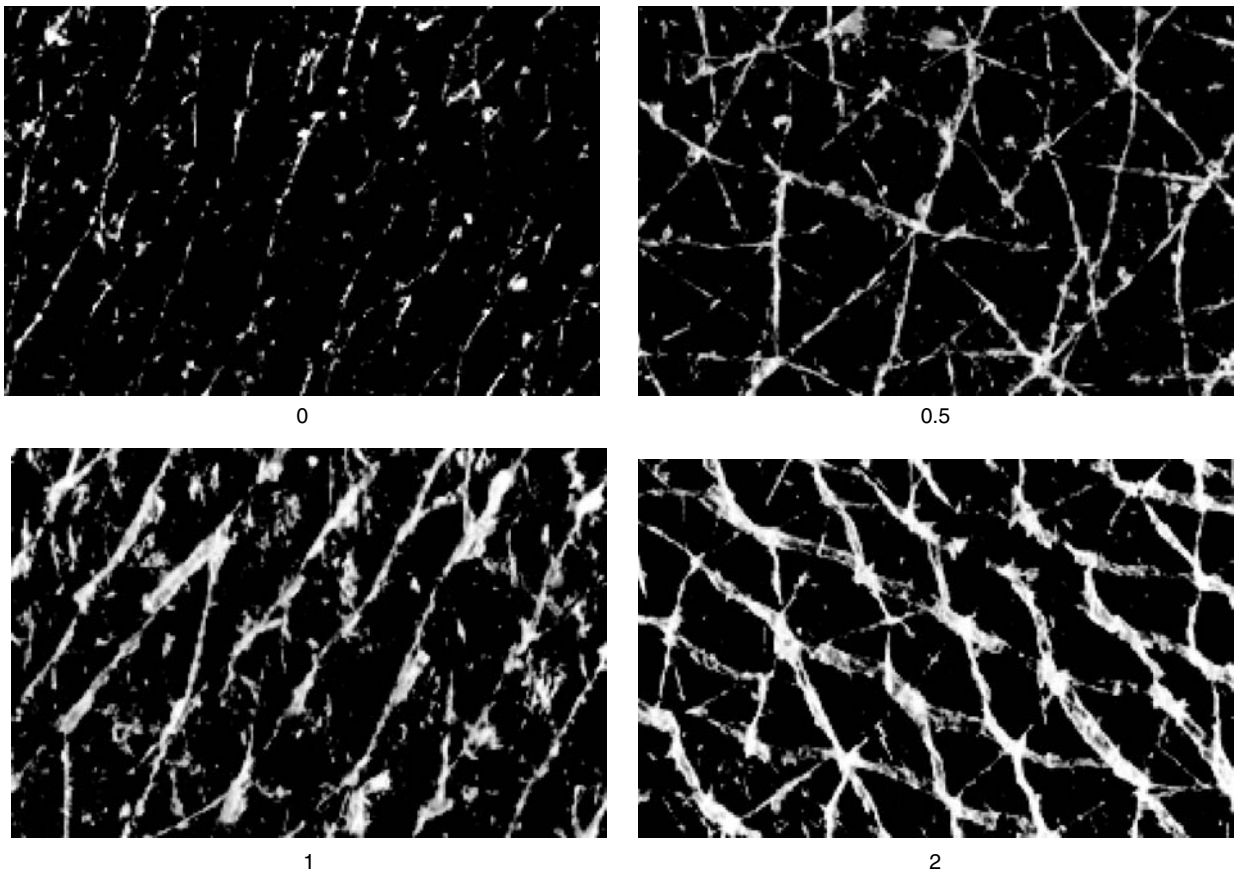


FIGURE 45.11 Scoring system for deepened skin furrow. The skin reactions were scored according to the degree of appearance of deepened skin furrows. No skin furrow change: 0, skin furrows which were deepened up to 25% of the area of the sample: 0.5, from 25 to 50%: 1, for more than 50%: 2.

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46 Analysis of Structural Change in Intercellular Lipids of Human Stratum Corneum Induced by Surfactants: Electron Paramagnetic Resonance (EPR) Study

Yoshiaki Kawasaki, Jun-ichi Mizushima, and Howard I. Maibach

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46.1 INTRODUCTION

The human skin is the largest organ in the body and serves the major function of protecting the underlying tissues from external elements. The skin offers a formidable barrier in the form of a multilayered stratum corneum, which is renewed continuously by the underlying epidermis. With increasing use of cosmetics and cleansing products, the human skin is brought into contact with the variety of excipients used in these topical formulations. Many of these contain surfactants, which can have toxic and irritating effects on skin. In addition, these amphiphilic molecules can partition into the stratum corneum and compromise the epidermal barrier function.

The intercellular lipid lamellae in the stratum corneum constitute the main epidermal barrier to the diffusion of water and other solutes (Elias and Friend, 1975; Elias, 1981, 1983; Wertz and Downing, 1982; Landman, 1986). These lipids, arranged in multiple layers between the corneocytes (Swartzendruber et al., 1989; Wertz et al., 1989), consist of ceramides (40–50%), free fatty acids (15–25%), cholesterol (15–25%), and cholesterol sulfate (5–10%) (Swartzendruber et al., 1987; Gray et al., 1982; Long et al., 1985). Information on the molecular structure of these lipids is important in elaborating a rational design for effective penetration enhancers in transdermal drug delivery (Woodford and Barry, 1986) and to understand the mechanism of irritant dermatitis and other stratum corneum diseases. This information has been obtained by thermal analysis (Van Duzee, 1975; Golden et al., 1987a; Bouwstra et al., 1992), x-ray diffraction study (Vilkes et al., 1973; White et al., 1988; Bouwstra et al., 1991a,b; Garson et al., 1991;

Bouwstra et al., 1994), FTIR spectroscopy (Bommannann et al., 1990; Krill et al., 1992), and electron paramagnetic resonance spectroscopy (EPR) (Rehfeld et al., 1988, 1990).

Several investigators (Imokawa et al., 1975; Faucher and Goddard, 1978; Imokawa, 1980; Fulmer and Kramer, 1986; Rhein et al., 1986, 1990; Froebe et al., 1990; Barker et al., 1991; Giridhar and Acosta, 1993; Wilmer et al., 1994) have demonstrated that stratum corneum swelling, protein denaturation, lipid removal, inhibition of cellular proliferation, and chemical mediator release contribute to irritation reactions. However, the mechanism of irritant dermatitis has not yet been understood and defined completely.

Whereas, permeability is increased by an increase in fluidity both in biological and artificial membranes, suggesting a correlation between flux and fluidity (Knutson et al., 1985; Golden et al., 1987b). The dynamic properties of intercellular lipids in the stratum corneum are incompletely characterized; the effect of surfactants has not been studied in detail.

Electron paramagnetic resonance (EPR) employing nitroxide spin probes, known as the spin labeling method, has been utilized as a valuable spectroscopic method for providing information about the dynamic structure of membranes (Sauerheber et al., 1977; Curtain and Gorden, 1984). Spin probes are specifically incorporated into the lipid or lipid part of biological membranes. Thus, each label reflects the properties of different membrane regions. EPR spectra of membrane-incorporated spin probes are sensitive to the rotational mobility and orientation of the probes, and to the polarity of the environment surrounding the probes.

In this chapter, the influence of surfactants on the intercellular lipid structure of cadaver stratum corneum and the stripped stratum corneum will be discussed, based on spin-label EPR spectroscopy. Techniques used to investigate fluidity of intercellular lipid layers of human stratum corneum will also be reviewed. In the meantime, we will also show the correlation between EPR spectral data and human clinical data such as transepidermal water loss (TEWL).

46.2 WHAT IS ELECTRON PARAMAGNETIC RESONANCE SPECTROSCOPY?

EPR, also known as electron spin resonance (ESR), is the name given to the process of resonant absorption of microwave radiation by paramagnetic ions or molecules, with at least one unpaired electron spin in the presence of a static magnetic field. EPR was discovered by Zavoisky in 1944. It has a wide range of applications in chemistry, physics, biology, and medicine: it may be used to probe the "static" structure of solid and liquid systems, and is also very useful in investigating dynamic processes.

Most biological systems give no intrinsic EPR signal because they have no unpaired electrons. Therefore, if EPR is to be used in studying these systems such as lipid membranes or macromolecules, one or more radicals known as spin labels must be coupled to the system under investigation. The spin label thus is an extrinsic probe or reporter group providing information that reflects the state of the biological system.

46.2.1 Principles

The detailed principles of EPR are explained in the book written by Wertz and Bolton (1972). Here, EPR principles are introduced in brief.

The principles of EPR are similar to those of NMR (nuclear magnetic resonance). The magnetic moment of an unpaired electron is given by

$$m = -g_e(eh/4\pi m_e)m_s(\mathrm{SI})$$

in which g_e is the electronic g-factor (a number very nearly equal to 2). $-e$ and m_e are, respectively, the electronic charge and mass, and m_s the spin quantum number (equal to $\pm 1/2$). The quantity in parentheses in the first equation is called the *Bohr magneton* and has the value of 9.2732×10^{-24} J T^{-1} in SI units (9.2732×10^{-21} erg G^{-1}). In an applied magnetic field of strength B, the transition of an electron from ground to the excited state requires energy:

$$\Delta E = g_e(eh/4\pi m_e)B(\mathrm{SI})$$

In a magnetic field of 2 T (20 kG), this energy corresponds to the absorption of radiation of the frequency:

$$\upsilon = \Delta E/h = 2\times(9.273\times10^{-24}\,\mathrm{J\,T^{-1}})\,(2\,\mathrm{T})/6.62\times10^{-34}\,\mathrm{Js}$$

$$= 5.6\times10^{10}\,\mathrm{Hz}$$

which is in the microwave region of the spectrum.

Paramagnetic substances are detected readily by EPR. About 10^{-13} mole of a substance gives an observable signal, so this technique is one of the most sensitive of all spectroscopic tools (Eisenberg and Crothers, 1979).

The effect of a neighboring nuclear spin on the resonance of an unpaired electron is called hyperfine coupling. For an electron in a magnetic field, there are two orientations and two quantum states (Figure 46.1a), giving a possible combination of four quantum states for the electron–nucleus pair. Nuclear spin "splits" each electron quantum state into two states. Because the selection rules for transitions in hyperfine coupling are $\Delta m_s = \pm 1$ and $\Delta m_I = 0$, there can only be two transitions among these four states for which electromagnetic radiation can be absorbed. Vertical arrows in Figure 46.1b show these two transitions.

For the ^{14}N nucleus, for example, $I = 1$, so there are three nuclear spin quantum states. Thus, a nearby ^{14}N nucleus splits the electronic levels into six levels. Three transitions are allowed among the six levels; consequently, the spectrum consists of three absorption bands.

46.2.2 Electron Spin Resonance Spectrometer

A modern ESR instrument consists of three basic units: (1) a microwave bridge and resonator, (2) a variable field magnet, and (3) signal amplification circuitry (Figure 46.2).

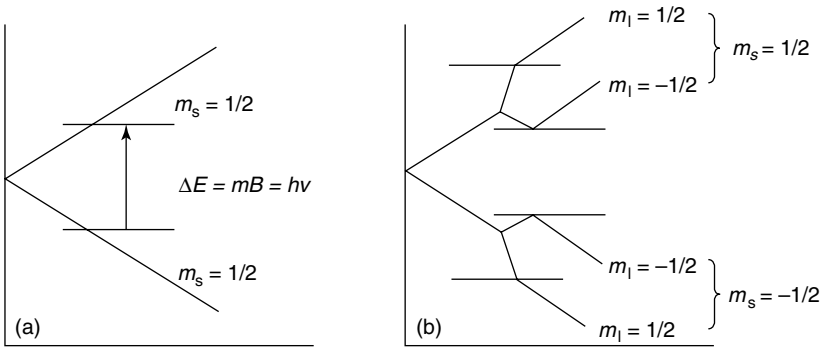


FIGURE 46.1 Energy levels of an unpaired electron with spin quantum states $m_s = 1/2$: (a) in a magnetic field; and (b) in a magnetic field and coupled to a nuclear spin of $I = 1/2$, with nuclear spin quantum.

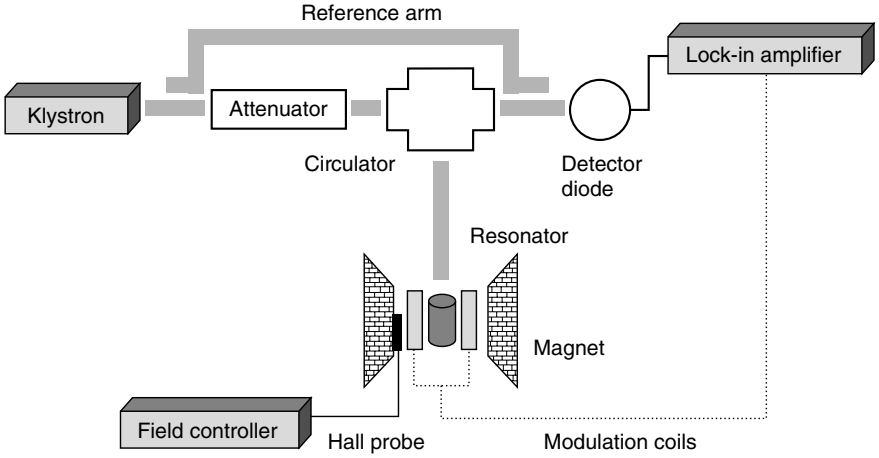


FIGURE 46.2 Block diagram of a typical EPR spectrometer.

Microwaves of the desired frequency are generated by either a klystron or Gunn diode. Their intensity is adjusted by an attenuator and transmitted via a waveguide to the sample chamber/resonator. During resonance, a small amount of microwave is reflected from the resonator and detected by a Shottky diode. To separate the reflected and incident microwaves, a circulator is placed between the attenuator and resonator. The circulator channels the microwaves in a forward direction: incident microwaves to the resonator and reflected microwaves to the detector. The bridge often contains an additional pathway—a reference arm which taps off a small fraction of the microwaves from the source—which bypasses the resonator and falls onto the detector to ensure its bias for the optimal detection of small intensity changes during resonance.

A static magnetic field is provided by an electromagnet stabilized by a Hall probe. The field is slowly swept by varying the amount of current passing through the electromagnet.

46.2.3 Spin Labeling Method; Paramagnetic Nitroxide Molecules That Serve as Probes in Membranes

McConnell et al. (1972) showed that significant information could be derived about macromolecules and membranes

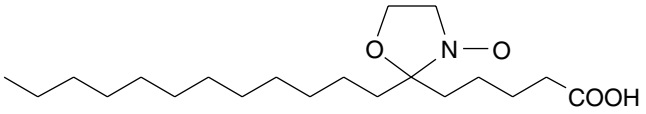


FIGURE 46.3 Chemical structure of spin probe; 5-Doxyl Stearic Acid (-DSA).

from the EPR spectra of bound nitroxide molecules. These are stable molecules that possess an unpaired $2p$ electron. The unpaired electron endows the molecules with strong EPR spectra. 5-Doxyl stearic acid (5-DSA) is one of the most commonly used spin probes and its structure is shown in Figure 46.3. A nitroxide molecule bound to a macromolecule is called a spin label.

Because the ^{14}N nucleus in a nitroxide molecule is near the unpaired electron, there is an interaction between them, thereby producing hyperfine splitting in the EPR spectrum. The ^{14}N nucleus has a spin of one, and consequently three absorption bands appear in the EPR spectra. The EPR spectra are usually recorded as the first derivative of the absorption spectrum, so instead of three bands there are three rise-and-dip spikes, which are the derivatives of the three bands. Triplet signals, which are sharp, can be observed when the spin probe moves freely, as shown in Figure 46.4. However, the spectrum becomes broader when spin probe mobility is restricted by interaction with other

components. When the spin probe is incorporated into the highly oriented intercellular lipid structure of normal skin, the probe cannot move freely due to the rigidity of the lipid structure, and its EPR spectrum represents the broad profile as seen in Figure 46.5a. Once the normal structure is completely destroyed by chemical or physical stress, there is nothing to inhibit probe mobility, and the EPR spectrum profiles become sharp, as in Figure 46.5b. The EPR spectral profile represents the rigidity of the environment of the spin probe. To express the rigidity quantitatively, an order parameter S is calculated from the EPR spectrum.

Spin labels provide information about the molecules to which they are bound. They can report the rate of motion of the molecule to which they have been covalently bound, or the amount of thermal motion in a membrane into which they have been inserted. The principle is that the bands of the EPR spectrum are broadened when the spin label is immobilized and narrowed when it is tumbling rapidly. The narrowing comes from the more rapid relaxation of the spin when neighboring groups are moving rapidly with respect to the spin label.

A second type of information is the polarity of the local environment surrounding the spin probe. The extent of splitting of the side bands from the central band depends on the dielectric constant of the medium in which the spin label is dissolved. Solvents of high dielectric constant augment the polarity of the N–O bond and increase the splitting. By measuring the splitting, an estimate can be made of the polarity of the surroundings of the spin label. This is of interest, for example, when a spin label is bound to a membrane, since it allows one to determine if the label is bound near the polar head groups or near the nonpolar hydrocarbon chains (Mehlhorn and Keith, 1972).

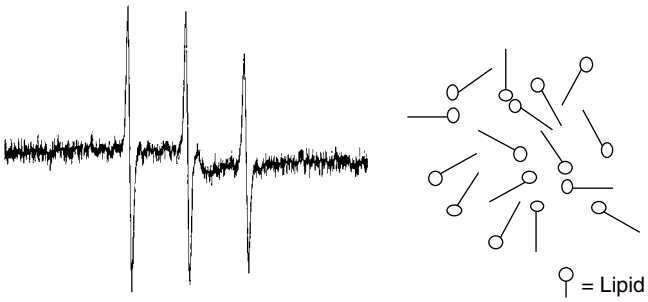


FIGURE 46.4 EPR spectrum of 5-DSA in aqueous solution.

46.2.4 How to Read the EPR Spectrum; Calculation of Order Parameter *S*

Order parameters were calculated according to Griffith and Jost (1976), Hubbel and McConnell (1971), and Marsh (1981):

$$S = (A\,||\,-A\,\perp)/[A_{ZZ} - 1/2(A_{XX} + A_{YY})](a_0/a_0')$$

where 2 A|| is identified with the outer maximum hyperfine splitting A_{max}, and A⊥ is obtained from the inner minimum hyperfine splitting A_{min} (Figure 46.5).

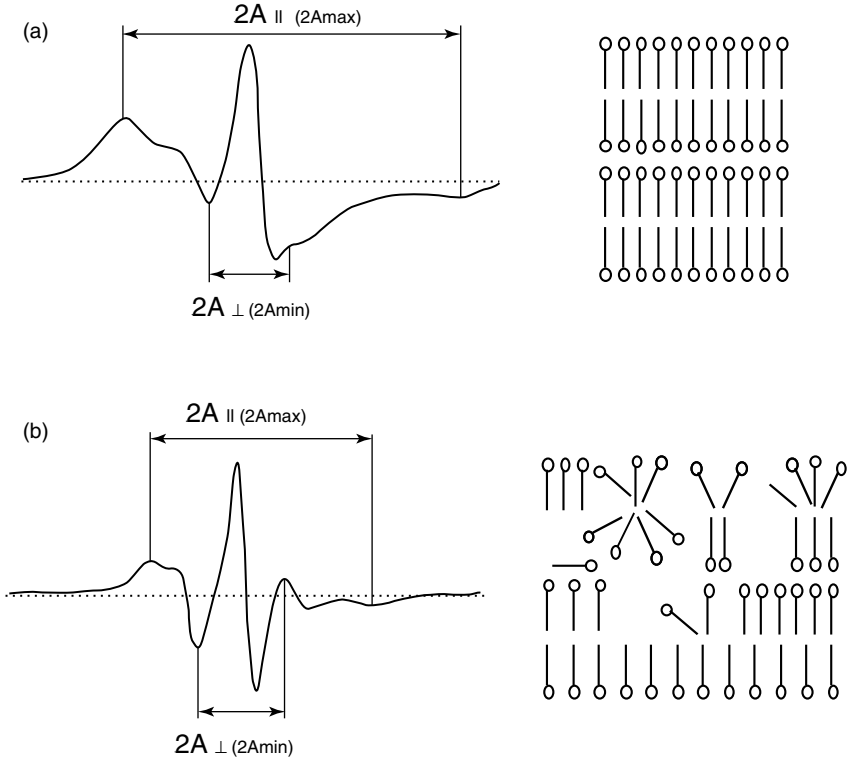


FIGURE 46.5 EPR spectrum of 5-DSA labeled stratum corneum from cadaver, (a) nontreatment (control), (b) treated with 1%wt SLS (sodium lauryl sulfate) for 24 h.

a_0 is the isotropic hyperfine splitting constant for nitroxide molecules in the crystal state.

$$a_0 = (A_{XX} + A_{YY} + A_{ZZ})/3$$

The values used to describe the rapid anisotropic motion of membrane-incorporated probes of the fatty acid type are

$$(A_{XX}, A_{YY}, A_{ZZ}) = (6.1, 6.1, 32.4) \text{ Gauss}$$

Similarly the isotropic hyperfine coupling constant for the spin label in the membrane (a_0') is given by

$$a_0' = (A \,||\, + 2A \perp)/3$$

a_0' values are sensitive to the polarity in the environment of the spin labels since increases in a_0' value reflect an increase in the polarity of the medium.

The order parameter provides a measure of the flexibility of the spin labels in the membrane. It follows that $S = 1$ for highly oriented (rigid) states and $S = 0$ for completely isotropic motion (liquid). Increases of order parameter reflect decreases in the segmental flexibility of the spin label, and conversely decreases in the order parameter *S* reflect increases in the flexibility (Curtain and Gorden, 1984).

46.3 EXPERIMENTAL DESIGN

46.3.1 EPR Measurement of Stratum Corneum from Cadaver; Spin Labeling and Surfactant Treatment Procedure

Human abdominal skin was obtained from a fresh cadaver with a dermatome. Epidermis was separated from dermis by immersing the skin in a 60°C water bath set for 2 min followed by mechanical removal. Then, the epidermis was placed, stratum corneum side up, on the filter paper and floated on 0.5%wt trypsin (type II; Sigma) in a Tris-HCl buffer solution (pH 7.4) for 2 h at 37°C. After incubation, any softened epidermis was removed by mild agitation of the stratum corneum sheet. Stratum corneum was dried and stored in a desiccator at –70°C for 3–4 days. Details are described by Quan (Quan and Maibach, 1994; Quan et al., 1995).

One slice of dry stratum corneum sheet (approximately 0.5 cm^2; ~0.7 cm × ~0.7 cm) was incubated in a 1.0 mg/dL 5-DSA aqueous solution (2.6×10^{-5} M; FW = 384.6) for 2 h at 37°C and washed gently with deionized water to remove the excess spin label.

Surfactant treatment was as follows: a spin-labeled section of stratum corneum was immersed in surfactant aqueous solution and incubated at 37°C for 1 h. The stratum corneum was taken out of the surfactant solution at indicated times. After rinsing with deionized water and removing the excess water, the stratum corneum was mounted on a flat surface EPR cell and EPR spectra were recorded.

The control EPR spectrum was recorded for the spin-labeled section of stratum corneum kept in the deionized water at 37°C instead of that kept in the surfactant solution.

46.3.2 EPR Measurement of Stripped Human Stratum Corneum; Spin Labeling and Surfactant Treatment Procedure

Two hundred microliters of aqueous solution of 1.00%wt surfactants was applied to the midvolar forearm using occlusive polypropylene chambers (1.8 cm diameter; Hilltop Laboratory, Cincinnati, OH, USA) for 24 h. Deionized water served as vehicle controls. Application sites for the different treatments were rotated to avoid an anatomical selection bias (Van der Valk and Maibach, 1989; Cua et al., 1990; Lee et al., 1994). Each site was examined visually by the same investigator and using following instrumental methods: TEWL, electrical conductance, and chromametry.

After these noninvasive measurements, patch site stratum corneum was removed from the volar side of the forearm skin by a single stripping with one drop of cyanoacrylate resin onto a quartz glass in accordance with the method of Imokawa et al. (1991). Stripped stratum corneum attached to a quartz glass was spin labeled with a drop (approximately 30 μL) of 1.0 mg/dL 5-DSA solution for 30 min at 37°C, then washed with deionized water to remove excess spin probe on the stripped skin surface.

Stripped stratum corneum was attached to a quartz cell, and EPR measurement was similarly conducted for cadaver stratum corneum.

46.4 RESULTS AND DISCUSSION

46.4.1 Effect of Surfactants on the Intercellular Lipid Fluidity of Cadaver Stratum Corneum

Kawasaki et al. (1995, 1997, 1999) and Mizushima et al. (2000) have examined the influence of surfactants on human stratum corneum obtained from cadaver (Table 46.1).

The surfactant molecule, which is amphiphilic to water and lipid, may be incorporated into structured lipids (lamellar structure). The order parameter calculated from 1.00%wt sodium lauryl sulfate (SLS)-treated stratum corneum was 0.47, indicating a disordering of the lipid structure. On the contrary, the high-order parameter value (0.73) for 1.00%wt monosodium lauryl glutamate (SLG) meant less of an effect on the structured lipid compared to the control-order parameter value (0.89). Treatment with 1.00%wt solution of SL, SLES, and SLEC revealed intermediate levels between SLG and SLS. Lipid disorder induced by MSAC and HEA, which are classified into a category different from anionic surfactants, also revealed intermediate levels between SLS and SLG. Note that 1.00%wt MSAC and 1.00%wt HEA, which are quaternary and amphoteric compounds, respectively, lead to less disorder in lipid structure than 1.00%wt SLS, although the irritation potential of surfactants is widely assumed to

follow the given pattern in which quaternaries are the most irritating; quaternaries > amphoterics > anionics > nonionics (Rieger, 1997). These two compounds (1.00%wt MSAC and 1.00%wt HEA) have plus charges. Their interaction with stratum corneum may be different from that of anionics such as SLS. A plus charge might have more attractive interaction with proteins electrically because proteins are generally thought to be negatively charged.

The change of order parameter corresponds to the structural changes in lipid layers. We can speculate that there are two phases in the increase of fluidity in lipid structure (decreasing the order parameter). The first phase is an effect of surfactants incorporated into the lamellar structures. If the surfactant interferes with or decreases lateral interactions between lipids, mobility increases in a way similar to the phase conversion from liquid crystal to gel in the lamellar layers. The second phase is the destruction of lamellar structure by micellization or solubilization of lipids by the surfactant. In this case, lipids no longer have dimensional restrictions and gain much higher mobility.

TABLE 46.1
Order Parameters of Stratum Corneum Treated with Surfactants and Clinical Observations

Category	Sample Name	Concentration (%wt)	Order Parameter *S*	Visual Scores (Average ± SD)	Visual Scores (Average ± SD) g/m²/h
Control	Water		0.89 ± 0.04	0.00 ± 0.00	5.0 ± 1.1
Anionic	SLS (Sodium lauryl sulfate)	1.0	0.47 ± 0.05	0.79 ± 0.30	13.6 ± 3.1
	SL (Sodium laurate)	1.0	0.65 ± 0.06	0.08 ± 0.20	7.1 ± 3.9
		5.0	0.52 ± 0.04	0.67 ± 0.30	13.3 ± 3.7
	SLES (Sodium lauryl POE (3) ether sulfate)	1.0	0.62 ± 0.06	0.42 ± 0.30	7.6 ± 2.8
	SLEC (Sodium lauryl POE (3) ether carboxylate)	1.0	0.62 ± 0.05	0.08 ± 0.20	7.4 ± 2.9
	SLG (Monosodium lauryl glutamate)	1.0	0.73 ± 0.07	0.04 ± 0.10	6.7 ± 3.5
		5.0	0.77 ± 0.08	0.13 ± 0.20	5.5 ± 2.3
Cationic	MSAC (Monostearylammonium chloride)	1.0	0.68 ± 0.02	NA	10.2 ± 1.9
Amphoteric	HEA	1.0	0.75 ± 0.02	NA	9.2 ± 0.8

Note: NA: visual grading is different from that of anionics.

TABLE 46.2
EPR Spectral Data and Clinical Data of SLS/SLG Mixtures

Sample Name	Averaged Order Parameter (Mean ± SD; $n = 3$)	Human Patch (Mean ± SD)	
		Visual Score	TEWL (g $H_2O/m^2/h$)
Control	0.86 ± 0.03	0.53 ± 0.08	13.0 ± 1.0
0.25%wt SLS	0.70 ± 0.02	0.73 ± 0.08	22.3 ± 1.7
0.50%wt SLS	0.66 ± 0.04	0.70 ± 0.10	22.3 ± 1.7
0.75%wt SLS	0.64 ± 0.03	0.87 ± 0.14	22.7 ± 1.5
1.00%wt SLS	0.56 ± 0.03	1.03 ± 0.15	25.4 ± 2.6
0.25%wt SLS + 0.75%wt SLG	0.81 ± 0.07	0.42 ± 0.30	20.0 ± 1.7
0.50%wt SLS + 0.50%wt SLG	0.71 ± 0.00	0.08 ± 0.20	20.7 ± 1.9
0.75%wt SLS + 0.25%wt SLG	0.66 ± 0.04	0.04 ± 0.10	21.2 ± 2.6
0.25%wt SLS + 1.00%wt SLG	0.81 ± 0.05	NA	NA
0.50%wt SLS + 1.00%wt SLG	0.79 ± 0.05	NA	NA
0.75%wt SLS + 1.00%wt SLG	0.74 ± 0.04	NA	NA
1.00%wt SLS + 1.00%wt SLG	0.66 ± 0.05	NA	NA
1.00%wt SLG	0.82 ± 0.02	0.67 ± 0.08	15.8 ± 1.1

Note: Error bars: mean ± SD, $n = 3$ for order parameters, mean ± SD, $n = 15$ for clinical data; NA: not available in Imokawa et al. (1991).

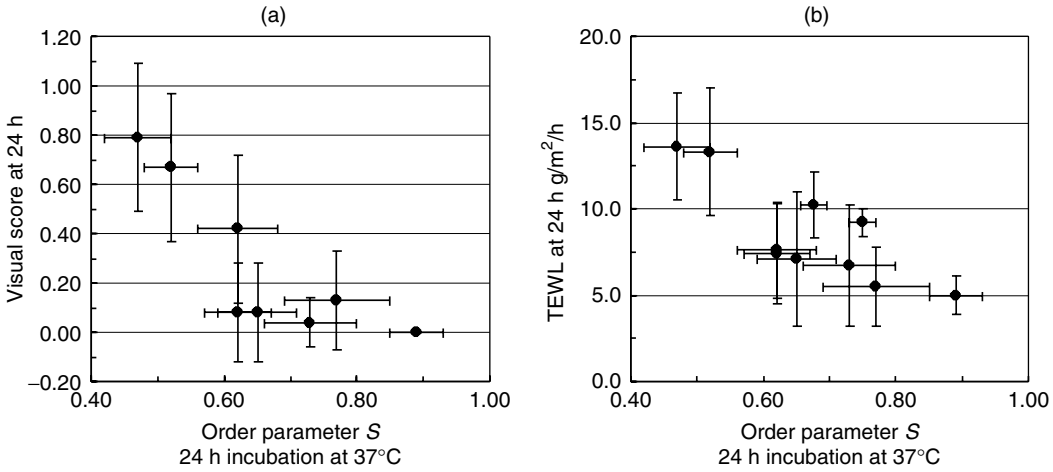


FIGURE 46.6 Correlation between clinical data of 24 h patch and order parameter *S* of 5-DSA labeled cadaver stratum corneum incubated in surfactant solution for 1 h at 37°C: (a) Correlation between order parameters and visual scores; (b) Correlation between order parameters and TEWL (error bars: mean ± SD, $n = 3$ for order parameter; mean ± SD, $n = 14$ for clinical data).

The results shown in Table 46.2 indicate that mobility increase induced by SLG can be attributed to phase-one structural changes in the lipid layers and that SLS might cause further disruption of the structures of lipid layers. The role of water in the stratum corneum must also be considered in an examination of the effects of surfactants on lipid layers. Treatment with anionic surfactants might influence water penetration and skin swelling (Takino et al., 1996). Rhein et al. (1986, 1990) examined the swelling of stratum corneum caused by surfactants and reported that the swelling effect of surfactants suggests a mechanism of action as the basis for *in vivo* irritation potential.

Figure 46.6 shows the correlation between the order parameter obtained from an EPR spectrum and the clinical readings. The correlation coefficients (r^2) of visual score and TEWL values were 0.76 and 0.83, respectively. The order parameter correlates to TEWL values better than to visual scores. This difference may be explainable in that TEWL is a direct measure of water barrier function, while visual scores represent total skin reactions including physical or structural changes of skin tissue due to physiological or biological reactions with surfactants. The visual score and colorimetry showed similar correlation coefficients, which mainly reflect reactions of the skin including edema of the epidermis and upper dermis, perivascular infiltrates, and vasodilation. The order parameter might not predict subsequent skin reactions after a disorder of the lipid structure caused by the denaturation of proteins or mucosaccarides in the dermis.

Order parameter measurement of stratum corneum may predict the minimal difference in irritation potential among a range of surfactants.

46.4.2 Effect of Surfactant Mixtures (SLS/SLG) on Intercellular Lipid Fluidity of Cadaver Stratum Corneum

As discussed previously, SLS was the most severely irritating and SLG the mildest amongst the anionic surfactants tested. Kawasaki et al. (1999) examined the influence of surfactant mixtures (SLS/SLG) on the intercellular lipid fluidity of stratum corneum obtained from cadaver skin.

The order parameter of water-treated stratum corneum (vehicle control) was 0.86 ± 0.03. Anionic surfactants as amphiphilic molecules may be incorporated into structured lipids (the lamellar structure). The order parameter calculated from 1.00%wt SLS-treated stratum corneum was 0.56 ± 0.03, indicating disorder in the lipid structure. On the contrary, the high-order parameter value (0.82 ± 0.02) for 1.00%wt SLG meant that less lipid structure was disordered; 1.00%wt SLG was almost equal to water. Treatment with 0.25%wt, 0.50%wt, and 0.75%wt SLS solutions revealed intermediate levels between 1.00%wt SLG and 1.00%wt SLS.

Each order parameter of 5-DSA labeled stratum corneum treated with SLS/SLG mixtures (the total concentration was constant at 1.00%wt) showed higher values than those of 0.25%wt, 0.50%wt, and 0.75%wt SLS, respectively. There were no statistically significant differences between 0.50%wt SLS and 0.50%wt SLS/0.50%wt SLG, and between 0.75%wt SLS and 0.75%wt SLS/0.25%wt SLG ($p > .05$). These profiles are also supported by the results of Kanari et al. (1993).

These results suggest that SLG inhibited SLS-induced lipid fluidization. To confirm the antifluidization of SLG, SLS/SLG mixture solutions were prepared with the SLG concentration constant at 1.00%wt, and 5-DSA labeled stratum corneum was treated with them. Then the EPR spectra were measured. The calculated order parameters are plotted in Figure 46.7.

Order parameters at each SLS concentration (0.25, 0.50, 0.75, and 1.00%wt SLS) with 1.00%wt SLG showed higher values than those of SLS-only solutions. There were statistically significant differences between solutions with and without 1.00%wt SLG ($p < .05$), suggesting that the addition of 1.00%wt SLG inhibits the fluidization of intercellular lipids induced by SLS. It may be hypothesized that the direct interactions between SLS and intercellular lipids would be interrupted by SLG and the log *P* (partition coefficient;

log {$[SLS]_{lipid}/[SLS]_{bulk}$}) of SLS into the intercellular lipid may be decreased.

The role of water in the stratum corneum must also be considered in analyzing the effects of surfactants on lipid layers. Alonso et al. (1995, 1996) reported that water increases the fluidity of the intercellular lipids of rat stratum corneum in the region closer to the hydrophilic area but not in the lipophillic area, deep inside the intercellular lipid layer.

The order parameter correlated to the clinical readings (Figure 46.8). The correlation coefficients (r^2) of visual score and TEWL values were 0.73 and 0.83, respectively. The order parameter correlates to TEWL values better than to visual scores, which is same as the result shown in the previous section.

The order parameters represent the disorder of stratum corneum induced by short-term surfactant contact. However, the clinical data represent skin irritation reactions induced by 24-h occlusive contact with surfactants. Order parameter measurement of stratum corneum may predict the minimal difference in irritation potential among a range of surfactants.

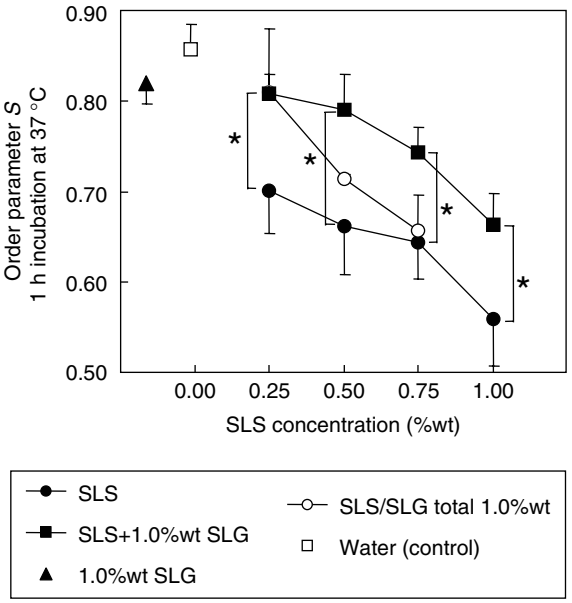


FIGURE 46.7 Order parameter of 5-DSA labeled cadaver stratum corneum treated with water, SLS, SLG, and SLS/SLG mixtures (total concentration 1.00%wt, 1.00%wt SLG addition to the SLS solutions; error bars: mean ± SD, $n = 3$; * indicates that $p < .05$).

46.4.3 Correlation Between CMC and Intercellular Lipid Fluidization for SLS

1.00%wt SLS causes more fluidization than other anionic surfactants. We still must ask: how long must there be contact with severe anionic surfactant SLS before fluidization happens in lipids? How much alteration is induced by how much concentration of SLS?

Figure 46.9 (unpublished data) shows the incubation time dependence of an EPR spectrum with different SLS concentrations. With increasing incubation time, the order parameter was decreased. However, each profile of incubation time dependence had a plateau at the region of 6 h and thereafter. The skin lipid alteration induced by SLS was typically completed within 6 h at a given concentration. However, each alteration level in intercellular lipids depended on its SLS concentration.

As the concentration of SLS increases, the order parameter at 24-h incubation decreases drastically in the range of 0–0.25%wt of SLS (Figure 46.10). However, the order parameters calculated from the stratum corneum treated with SLS at more than 0.5%wt had no significant difference, showing around 0.45~0.49. This critical point between 0.25 and 0.5%wt (8.7~17.3 mM) may correspond to the CMC (critical micelle concentration) for SLS at 37°C.

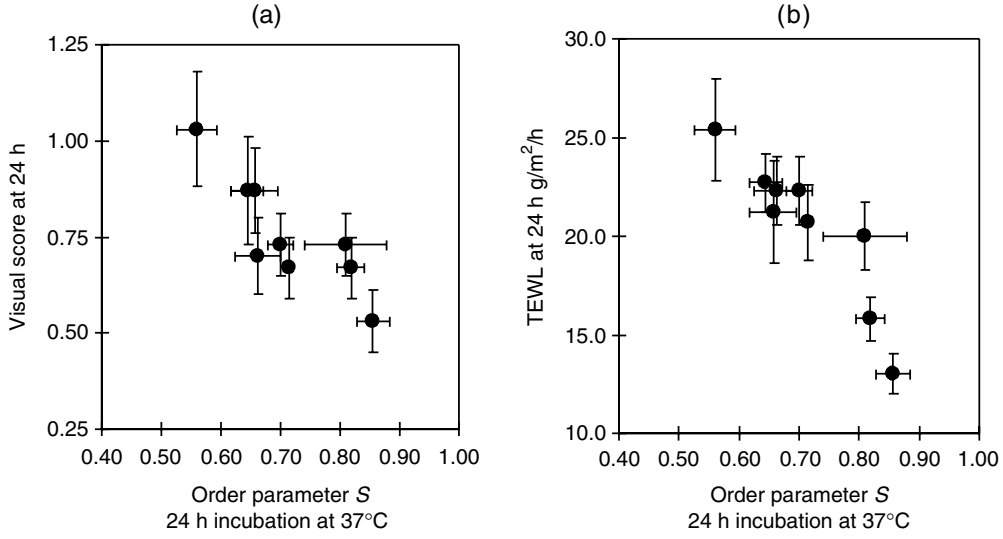


FIGURE 46.8 Correlation between clinical data of 24 h patch and order parameter *S* of 5-DSA labeled cadaver stratum corneum incubated in surfactant solution for 1 h at 37°C: (a) Correlation between order parameters and visual scores; (b) Correlation between order parameters and TEWL (error bars: mean ± SD, $n = 3$ for order parameter; mean ± SD, $n = 15$ for clinical data).

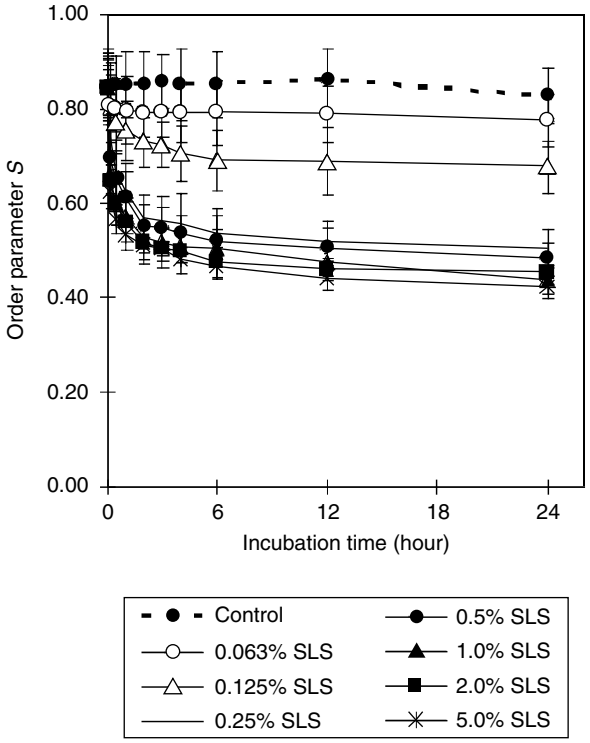


FIGURE 46.9 Time dependence of order parameters of 5-DSA labeled stratum corneum in SLS solution at various concentrations (error bars: mean ± SD, $n = 5$).

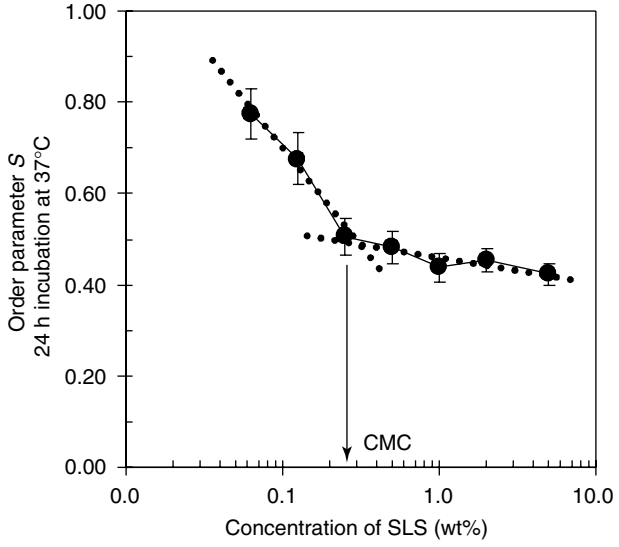


FIGURE 46.10 Correlation between SLS concentration and order parameters of 5-DSA labeled cadaver stratum corneum incubated at 37°C for 24 h (error bars: mean ± SD for $n = 5$).

Rosen (1978) reported that the CMC of SLS is 8.6 mM at 40°C and 8.2 mM at 25°C.

This behavior is consistent with the following general concerns among experts: monomeric surfactants can penetrate the skin. Monomeric molecules are also the species that are initially adsorbed into the various surfaces within the skin; we cannot ignore secondary bonding due to hydrophobic effects. Thus, the concentration of monomeric species probably plays a major role in skin and surfactant interactions (Rieger, 1995).

46.4.4 EPR Study Utilizing Human Stripped Stratum Corneum

All the previous data are based on human stratum corneum obtained from cadaver skin. To define the structural changes in intercellular lipids induced by the topical application of surfactants and to discuss the correlation between lipid alteration and skin irritation reactions, choosing human stratum corneum from cadavers for a substrate as a model site of skin irritation is much better than using animal skins such as those of guinea pigs and rats, or using lecithin liposomes. But stratum corneum is not sufficient for discussing the mechanism of irritant dermatitis. Cadaver stratum corneum is just a substrate, not a living system, which has a recovery system induced by signals such as chemical mediators. With the new procedure for measuring EPR spectra on human stripped stratum corneum, information on the dynamics of living skin may be provided.

Mizushima et al. (2000) examined EPR spectral data on stratum corneum from cadaver skin and stripped skin treated with three types of surfactants.

The correlation between order parameters of 5-DSA labeled cadaver stratum corneum treated with surfactants and those of 5-DSA labeled stripped stratum corneum was summarized in Figure 46.11.

Although the order parameters obtained from stripped stratum corneum are larger than those of cadaver stratum corneum, a high correlation between them is observed. It suggests that the order parameters of cadaver stratum

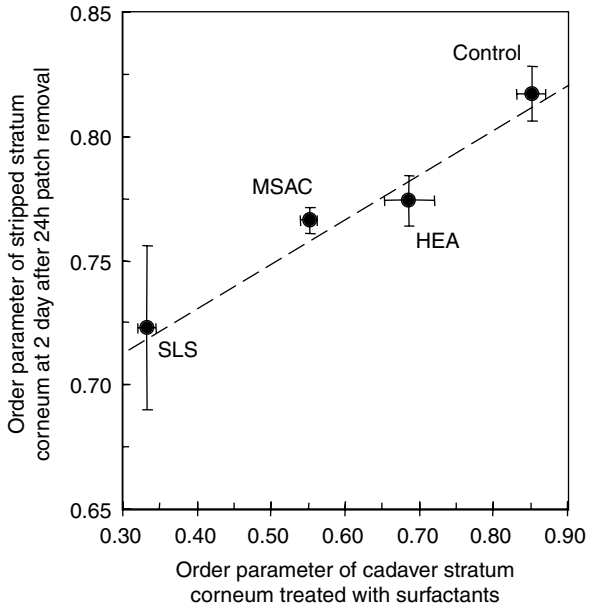


FIGURE 46.11 Correlation of order parameters between cadaver stratum corneum and stripped stratum corneum (error bars: mean ± SD for $n = 5$). Cadaver stratum corneum = −3.883 + 5.833 × stripped stratum corneum.

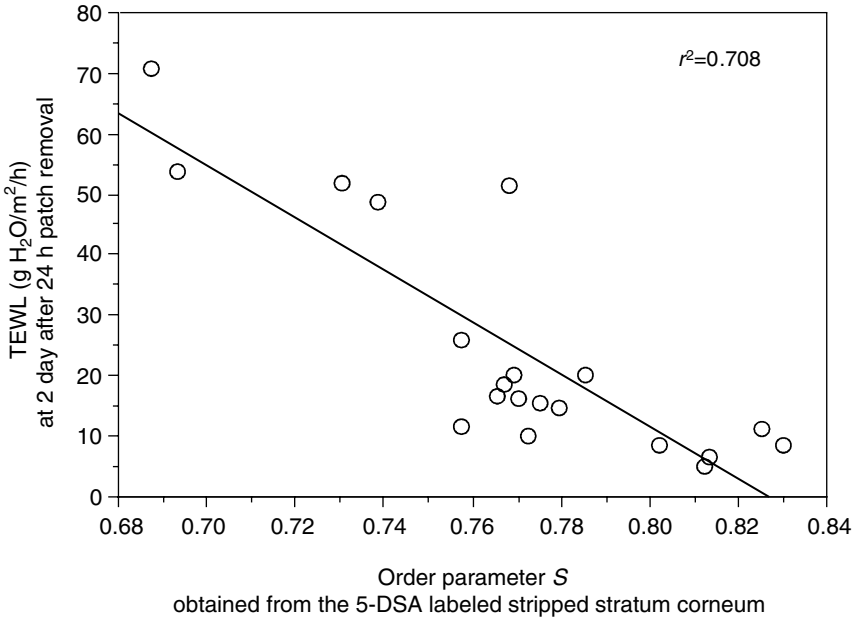


FIGURE 46.12 The correlation between TEWL and order parameters on the second day after 24 h patch removal.

corneum reflect the fluidity of the intercellular lipids in the irritaed skin.

The order parameter of SLS-treated cadaver stratum corneum is smaller than that of stripped stratum corneum. This difference may be due to the barrier-reconstruction property of skin itself. The epidermis can synthesize lipid immediately after barrier disruption (Grubauer et al., 1989). Skin barrier function was 80% repaired by 6–8 h, when skin was treated with acetone (Elias and Feingold, 1992).

The correlation between the order parameter of stripped stratum corneum and clinical readings is as follows: the correlation coefficient between order parameter and visual score, TEWL values on the second day after patch removal, were 0.526 and 0.708, respectively. The correlation with TEWL is high, as shown in Figure 46.12. This result consists of *in vitro* data based on cadaver stratum corneum.

46.4.5 Water May Affect the Order Parameter *S*

The role of water in the stratum corneum must be also considered for an understanding of the effects of surfactants on lipid layers. Alonso et al. (1995, 1996) reported that water increases the fluidity of intercellular lipids of rat stratum corneum at the region close to the hydrophilic area, but not in the lipophilic area, deep inside the intercellular lipid layer. Treatment with anionic surfactants may influence water penetration into stratum corneum. In case that the stratum corneum from a cadaver is treated with SLS and SLG for 1 h, the order parameter decreases with the dose dependency of the surfactants. When order parameter is followed over time under dry conditions, those values increase. After 24 h, the order parameter becomes higher than in the control (Kawasaki et al., 1997). It is suggested that the altering of water content in cadaver stratum corneum affects the order parameter. This does not simply mean loss of water, because after the untreated control stratum corneum dries, the order parameter still shows a minimal order parameter change.

To investigate such effects of surfactants, Mizushima et al. (2001) measured the EPR spectra on cadaver stratum corneum treated with four concentrations of SLS under wet conditions just after incubation. The EPR spectra were measured again on the same samples after they were dried at room temperature for 1 h. They also weighed each cadaver stratum corneum sample three times as follows: (1) before labeling (initial weight of each stratum corneum), (2) just after the treatment/EPR measurement of stratum corneum under wet conditions, and (3) after the second EPR measurement of stratum corneum under dry conditions.

As shown in Table 46.3, under wet conditions, the order parameter *S* decreased and the weight of each cadaver stratum corneum increased with SLS dose dependency. On the contrary, under dry conditions, the order parameter increased with the dose dependency of SLS compared to the untreated control, which meant that the fluidity of the stratum corneum increased greatly before surfactant treatment. The weight of each cadaver stratum corneum sheet decreased with SLS dose dependency. So we can say that the effect of SLS on stratum corneum is not only to alter the fluidity of the lipid bilayer, but also to change the water-holding capacity of the lipid bilayer. This might be due to depletion of or change in the intercellular lipid lamellae such as fatty acids, cholesterol derivatives, and materials, which cooperate with the keratin protein and amino acids as natural moisturizing factors.

Two phases can be hypothesized in the increase of fluidity in lipid structures. The first phase is in the effect of surfactants incorporated into the lamellar structures. If the surfactant interferes with or decreases lateral interactions between lipids, mobility increases in a way similar to the

TABLE 46.3
The Order Parameters *S* Obtained from SLS-Treated Cadaver Stratum Corneum ($n = 3$) and Its Weight, Measured under Wet and Dry Conditions

SLS Concentration (%wt)	Order Parameter		Weight Increase (%)	
	Wet Condition	Dry Condition	Wet Condition	Dry Condition
0.00	0.81 ± 0.01	0.82 ± 0.01	155.2 ± 4.3	95.2 ± 4.9
0.13	0.79 ± 0.01	0.83 ± 0.01	195.0 ± 9.8	87.1 ± 11.5
0.25	0.71 ± 0.01	0.84 ± 0.01	289.5 ± 47.7	82.5 ± 6.4
0.50	0.63 ± 0.02	0.84 ± 0.01	337.7 ± 28.8	81.6 ± 5.3
1.00	0.59 ± 0.01	0.84 ± 0.01	351.9 ± 21.3	80.3 ± 14.2

Note: The weight of the stratum corneum sheet is defined as 100 (%) just before labeling with 5-DSA aqueous solution.

phase conversion from liquid crystal to gel in the lamellar layers. The second phase is the destruction of the lamellar structure by means of micellization or solubilization of the lipid layer by the surfactant. In this case, lipids no longer have dimensional restrictions and gain much higher mobility. The surfactant might have changed the water-holding capacity of the stratum corneum, and the water content may change the fluidity of stratum corneum lipids. We have to consider the water content of stratum corneum because it will alter the fluidity of intercellular lipids. To evaluate the effects of chemicals such as surfactants on stratum corneum lipids, which may change the water-holding capacity, we have to consider the existing water content of stratum corneum in measuring the EPR spectra.

46.5 CONCLUSION

The toxic manifestations of topically applied substances may induce immediate phenomena (such as corrosion or primary irritation), delayed phenomena (such as sensitization), phenomena that require an additional vector (such as phototoxicity), and systemic phenomena (paraquat toxicity). Such reactions cannot occur unless the toxic agent reaches a viable part of the skin by going through the stratum corneum with accompanying intercellular lipid structure disruption. If the toxicant can be stored in or absorbed by a skin layer without any alteration in lipid structure, it may not reach the viable tissues at all or may be released relatively slowly, thus effectively prolonging the symptoms.

EPR spin labeling is a robust method for monitoring the structural change in intercellular lipids induced by topically applied surfactants. We have shown that order parameter is an easy to use and quantifiable method for predicting irritation reactions in the skin. In particular, EPR measurement on stripped stratum corneum may reflect the actual skin condition with regard to lipid structure. It may also aid in investigating the irritation potential of general chemicals, effects of topical penetration enhancers, drug delivery systems, and skin diseases such as xerosis and atopic dermatitis.

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47 Water and Saline Compresses in Treatment of Irritant Contact Dermatitis: Literature Review

Cheryl Y. Levin and Howard I. Maibach

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47.1 INTRODUCTION

The current armamentarium of therapies for irritant dermatitis includes application of cool compresses, topical corticosteroids, and occasionally oral corticosteroids. Although these treatments are "standard of care" in dermatologic practice, evaluation of their effectiveness using quantitative parameters has rarely been performed. This chapter will review the efficacy of deionized water or mineral compresses to treat experimentally induced irritant contact dermatitis (ICD). There are only three studies to date in the literature.

47.2 LITERATURE REVIEW

A study by Levin (2001) sought to determine the efficacy of both distilled water and physiologic saline compresses on experimentally induced ICD. 24 h application of both the lipophilic nonanoic acid (NAA) and the hydrophilic sodium lauryl sulfate (SLS) were used to induce ICD in nine healthy volunteers. Following irritation, compresses were applied twice daily for 30 min each for a total of four consecutive days. Transepidermal water loss (TEWL), laser Doppler flowmetry (LDF), chromametry, and visual scoring were used to quantify results. Cool compresses of both high performance liquid chromatography (HPLC)-grade deionized water and 3 mL of physiologic saline significantly reduced TEWL and LDF, with no statistically significant difference between the efficacy of the saline or water compresses. Chromametry and visual scoring did not detect a significant effect with either water or saline compresses. The results suggest improvement with daily application of either water or physiologic saline compresses in the treatment of acute ICD.

An experiment by Yoshizawa et al. (2001) observed that seawater components (including 500 mM NaCl) decreased TEWL to a greater extent than distilled water in volunteers with experimentally induced irritation. In his study, Yoshizawa openly applied 2% SLS for 10 min onto the volar forearms of three healthy volunteers. The irritation was followed by 20 min application of seawater, 500 mM NaCl, 10 mM KCl, 55 mM $MgCl_2$ or deionized water onto the irritated site. The SLS and subsequent water solutions were applied daily for 2 weeks, and the effects were measured with TEWL, a measure of epidermal barrier function, and capacitance—an indicator of stratum corneum water content. The seawater and NaCl significantly reduced TEWL and increased capacitance, while the KCl reduced TEWL but had no effect on capacitance. Furthermore, the distilled water and the $MgCl_2$ had no significant effect on TEWL or capacitance.

In another similarly designed study by Yoshizawa et al. (2003), three types of mineral water solutions, namely 500 mM NaCl + 10 mM KCl (solution A), 250 mM NaCl + 10 mM KCl (solution B), or 250 mM NaCl + 50 mM KCl (solution C), and deionized water were tested for their ability to treat experimentally induced subacute dermatitis of the volar forearm of three healthy subjects. First, 2% SLS was applied for 10 min, followed by 2 min application of the mineral water solutions. The SLS and subsequent mineral water solutions were applied daily for 11 days, and the effects were measured with TEWL, a measure of epidermal barrier function, and capacitance—an indicator of stratum corneum water content. Solution C statistically significantly inhibited the relative increase in TEWL and relative decrease in capacitance as compared to distilled water.

47.3 DISCUSSION

In these studies, NAA and SLS were used to model ICD. These irritants were selected because they are reproducible, easy to read, and irritate the skin without causing excessive discomfort to the volunteers (Wahlberg and Maibach, 1980; Lee and Maibach, 1995). Bioengineering and clinical parameters were utilized in both studies.

In Levin's study, compress application decreased quantitative measurements of both lipophilic (NAA)- and hydrophilic (SLS)-induced irritation, indicating their potential efficacy in treating irritation of two distinct physicochemical characteristics. In addition, Levin's study showed that TEWL and LDF, measures of skin barrier function and inflammation, respectively, are able to distinguish compress treatment from untreated control. However, two other parameters utilized, namely chromametry and visual scoring, did not. It seems, therefore, that the cool compresses accelerated the healing of underlying skin properties, while on the skin exterior, this healing process was not clearly observed.

Yoshizawa also showed that TEWL is a useful tool in assessing the efficacy of salt solutions. In addition, Yoshizawa assessed capacitance, an indicator of stratum corneum content. In Yoshizawa's studies, the salt solutions of either seawater or 500 mM NaCl inhibited stratum corneum dryness as measured by capacitance. As discussed in his article, the salt solutions' hygroscopic characteristics might enhance the water-holding capacity of the stratum corneum by natural moisturizing factor and sphingolipids.

The studies by Levin and Yoshizawa were designed differently and may not be easily compared. Yoshizawa used deionized water as a control and basis for comparison to the seawater and mineral water solutions, while Levin used deionized water and saline as compared to untreated control. In addition, Yoshizawa studied the effects of seawater and distilled water on cumulative ICD, while the study by Levin sought to determine the effects on acute irritant dermatitis. Distilled water may possess greater efficacy in treating acute ICD as compared with cumulative ICD. Additionally, differences in saline concentration, method, and duration of treatment application may also account for the discrepancy in the results of the two studies.

The mechanism by which saline and water compresses reduce irritation is not fully understood. Short applications of saline or water compresses may provide a moist environment for the healing of the irritation. In fact, water and saline have known hygroscopic characteristics and may therefore increase the capacity for intracellular moisture retention. Additionally, cool compresses may reduce both the inflammation and increased temperature associated with ICD. It seems plausible that the osmotic properties associated with the saline compresses may allow fluid to be drawn from the edematous lesions in some of the studies' subjects (Lim et al., 2000). In the experiment by Levin, postirritation skin sites were not washed with running water prior to compress treatment. Therefore, it is also possible that the water and saline compresses created a diluting effect, thereby decreasing the content of SLS on treated skin sites as compared to untreated sites. Certainly, the studies suggest that water and saline compresses improve the barrier function and reduce inflammation when applied postinduction of SLS or NAA irritation. Further exploration of working mechanisms for compress efficacy would be beneficial in guiding current dermatologist recommendations.

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48 Reaction of Skin Blood Vessels to Successive Insults on Normal and Irritated Human Skin

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48.1 INTRODUCTION

Various physiologic and pathologic processes, including the action of pharmacological substances, depend on histamine as a mediator. The response of irritated or otherwise altered skin to internal and external provocation may differ from the response of intact skin. The effect of histamine may depend on the timing of the sequence of insulting events. Tachyphylaxis, a refractory behavior of histamine receptors of the blood vessels, has been observed in clinical and laboratory studies, but quantitative documentation is lacking.

An attempt was made to demonstrate skin blood vessel reaction related to variations in the sequence of events. Following repeated insult, the reactivity of the cutaneous microvasculature to histamine was quantified with laser Doppler flowmetry (LDF). This noninvasive optical technique has been used to evaluate the response of the cutaneous microvasculature to challenge.[1,2]

48.2 MATERIALS AND METHODS

48.2.1 Subjects

Twenty healthy Caucasian volunteers, skin type II: 5 men and 15 women with an age range 21–44 years, average 36.9 years (6.5 S.D.). The subjects were not using any systemic or topical medication, including antihistamines or any other medication that might affect skin reaction to histamine. The subjects gave informed consent for the study, the protocol of which was approved by the University of California San Francisco (UCSF) Institutional Review Board.

48.2.2 Experimental

48.2.2.1 Irritation

Freshly prepared solutions of sodium lauryl sulfate (SLS), 1% in distilled water (Sigma Chemical Co, St. Louis, Missouri; 99% purity), were used as model water-soluble irritants. Subjects were patched with 12 mm Finn chambers (Epitest Ltd. Hyryla, Finland) by applying 50 μL of the solution on one site on the upper back for 24 h. Subsequently, this site received a single off-center intradermal histamine administration (off-center, so that subsequently LDF reading was taken inside the area of the SLS pretreated skin). Another site was similarly treated, but distilled water was used in the patch instead of SLS, in eight of the volunteers.

48.2.2.2 Intradermal Histamine Administration and Blood Flow Measurements

Histamine chloride (1 mg/mL, Extraits Allergeniques Stallergenes—Pasteur) was administered using prick-test needles (Stallerpoint, Stallergenes S.A. Cedex, France), with a point length of 1.0 mm. To ensure uniformity, all prick tests were applied perpendicularly by the same individual, using firm pressure for 20 s. First, baseline blood flow at each site was established, followed by intradermal (prick)

administration of histamine. The prick spot was marked on the skin. The reaction was recorded by LDF (laser Doppler blood flow monitor, MBF3D, Moor Instruments, Axminster, England, Acaderm, Menlo Park, California). Measurements started 2.5 min after histamine administration to avoid the transient increase in blood flow induced by the prick, which decays within 2 min.[3] On each subject, a control site on the back pricked with normal saline solution served to ensure that none of the subjects had an exaggerated reaction to the prick. All these control sites had to show a decay of the increase in blood flow within 2 min. A separate probe holder (PF 104, Perimed, Sweden) was positioned over each site and held in place with double adhesive disks (3M, St. Paul, Minnesota) and was left at the same spot for the duration of the experiment: Measurements at the center of the wheal are decreased and difficult to record because the extravasated fluid restricts blood supply at the site of histamine administration. Therefore, measurements were taken 1 cm from the point of histamine administration. A distance of 1 cm generated the most sensitive and reproducible data.[4,5] Measurements were taken by shifting the probe from one test site to another. For each subject, the prick test at the various time points was randomly assigned (computer-generated randomization), and the sequence of site measurements was random as well.

The sequence of provocations at each site is listed in Table 48.1.

All experiments were conducted in the same room under reasonably constant conditions (temperature 68–72°F, and relative humidity 40–60%) and during the same season (fall).

48.2.3 Characteristic Parameters

The response to histamine was characterized by four parameters: (i) the magnitude of the maximum response over the baseline value (P); (ii) the time required for the response to reach its maximum value (Tp); (iii) the time required for the maximum response to decay to half the maximum value (T1/2); and (iv) the area under the time-response curve from $t = 0$ to T1/2 (A1/2).

48.2.4 Statistics

Comparison between the parameters used to characterize the blood flow response (corrected for the baseline preadministration value) involved analysis of variance followed by the Newman–Keuls multiple comparison test.

48.3 RESULTS

Table 48.2 summarizes the response following histamine prick test. The peak response, P (8.22), and the extent of the response, A1/2 (114.21), of the second prick at site A (second prick done when the first prick response decayed to half its peak) were significantly higher than all the other responses at sites A, B, and C (range of P: 5.7–6.52, A1/2 ranged between 67.86 and 94.71) ($p < 0.01$). The peaks of response at sites A, B, and C did not significantly differ from each other.

The maximum response at site D (histamine prick was performed over SLS 24 h patch) was 12.88, significantly greater than at all other sites, which ranged between 5.7 and 8.22 ($p < 0.01$).

The time required to reach the peak (Tp) was similar at all test points, except for the irritated site (D), where two peaks resulted: the first one did not differ from the other points, and the second one followed later, at 25 min, significantly greater than all the other time points which ranged between 5.03 and 6.96 ($p < 0.02$).

The time to decay to half the maximum response was not different among the various injections and ranged between 13.49 and 17.72 min, except for the prick over irritated skin, where it was prolonged to 53.63 min ($p < 0.001$).

Table 48.3 summarizes the response following histamine prick test over the SLS and water patch sites. Histamine

TABLE 48.1
The Sequence of Provocations at the Various Sites

Site	Pretreatment	First Histamine Prick	Second Histamine Prick
A		$t = 0$	t = time for the first prick to decay to half peak response
B		$t = 0$	$T = 1$ h
C		$t = 0$	$T = 2$ h
D	SLS 1% 24 h (12 mm area of occlusion)	24 h	
E	Water patch 24 h	24 h	
F (control 1)		$t = 0$	
G (control 2)	SLS 1% 24 h (12 mm area of occlusion)		

Source: Tur, E., Tur, Z., Weltfriend, S., Schulze, K., and Maibach, H.I., *Exog. Dermatol.*, 1, 39–44, 2002. With permission.

TABLE 48.2
Laser Doppler Flowmetry (LDF) Response Following Histamine Prick Test

	A		B		C		D		
Site Parameter	$t = 0$	$t = t$ to 1/2 P	$t = 0$	$t = 1$	$t = 0$	$t = 2$	**SLS**	**Histamine $t = 24$**	**Histamine–SLS**
P[a,b]	6.39	8.22[a]	5.7	6.19	6.23	6.52	5.2	12.88	7.78[b]
SEM	(1.09)	(0.81)	(0.68)	(0.81)	(1)	(0.72)	(.6)	(1.5)	(1.5)
								2nd: 17.99	2nd: 12.9
								(1.7)	(1.7)
A1/2[a,b]	88.37	114.21[a]	94.71	77.96	67.86	73.25		585.97	307[b]
SEM	(28.54)	(17.72)	(30.43)	(26.34)	(12.95)	(12.87)		(119.1)	(89)
Tp (min)[c]	6.96	3.69	6.96	5.03	6.97	6.28		6.59	
SEM	(1.55)	(0.52)	(1.31)	(0.72)	(1.67)	(1.1)		(0.7)	
								2nd: 25[c] (2)	
T1/2[d] (min)	14.91	17.52	17.72	14.35	14.01	13.49		53.63[d]	
SEM	(3.1)	(2.13)	(3.46)	(2.89)	(2.11)	(2.0)		(5.13)	
Baseline	1.028	3.8	1.07	1.56	1.16	1.175	1.08		
	(0.047)	(0.44)	(0.08)	(0.3)	(0.05)	(0.06)	(0.05)		

Note: Values are means ± SEM for the 20 volunteers, corrected for the baseline. Laser Doppler flowmetry readings are expressed in arbitrary units.

[a] Both the peak response and the extent of the response as measured by the area (A1/2), of the second prick at site A (second prick done when the first prick response decayed to half its peak), were significantly higher than all the other peaks of response at sites A, B, and C ($p < 0.01$), which did not significantly differ from each other.

[b] The magnitude of the maximum response (P) and the extent of the response as measured by the area (A1/2) were significantly greater at site D, where histamine prick was performed over SLS 24 h patch than at all other pricks ($p < 0.01$).

[c] The time to the second peak at the irritated site (D) was longer ($p < 0.02$) than at all other pricks, which did not differ from each other.

[d] The time to decay to half the maximum response at the irritated skin (D) was longer ($p < 0.001$) than at all other pricks, which did not differ from each other.

Source: Tur, E., Tur, Z., Weltfriend, S., Schulze, K., and Maibach, H.I., *Exog. Dermatol.*, 1, 39–44, 2002. With permission.

TABLE 48.3
Laser Doppler Flowmetry (LDF) Response Following Histamine Prick Test Over the SLS and Water Patch Sites

Parameter	**Histamine–SLS[a]**	**W[a]**
P[a]	12.06	6.412
(SEM)	(3.2)	(1.72)
	(1st: 9.4 [2.9]	
	2nd: 16.4 [3.6])	
Tp[b] (min)	Sum: 14.8 (3.3)	8.44
(SEM)	1st: 7.1 (1.2)	(2.64)
	2nd: 29[b] (2.9)	
T1/2[c] (min)	53.7[c]	23.875
SEM	(5.3)	(6.23)
Baseline	1.08	1.07
SEM	(0.09)	(0.1)

Note: Values are means ± SEM for the eight volunteers. Laser Doppler flowmetry readings are expressed in arbitrary units.

[a] Histamine reaction over 24 h SLS patch (SLS readings subtracted) significantly higher than histamine reaction over 24 h water patch ($p < 0.04$).

[b] The time to the second peak at the irritated site (D) was longer ($p < 0.02$) than at the water patch site.

[c] The time to decay to half the maximum response at the irritated skin (D) was longer ($p < 0.001$) than the water patch site.

Source: Tur, E., Tur, Z., Weltfriend, S., Schulze, K., and Maibach, H.I., *Exog. Dermatol.*, 1, 39–44, 2002. With permission.

reaction over 24 h SLS patch (after subtraction of SLS readings) was significantly higher than histamine reaction over 24 h water patch ($p < 0.04$): 12.06 as compared to 6.41. The time to the second peak at the irritated site (D) was longer ($p < 0.02$) than at the water patch site: 29 min as compared to 8.44 min. The time to decay to half the maximum response at the irritated skin (D) was longer ($p < 0.001$) than the water patch site: 53.7 as compared to 23.87.

The characteristic response parameters are depicted in Figures 48.1 through 48.3.

48.4 DISCUSSION

Histamine skin reactions depend on many variables, even change of emotion can modify the reaction to histamine.[6] Clinical effects may vary with different combinations of repeated or combined exposure to various exogenous factors. Additivity or enhancement of the effect may occur, or the opposite—attenuation, as was shown for heat pain and scratching.[7] Identification of the relevant offending agent is difficult when dealing with an effect of a combination of factors, hence the importance of investigations of various situations. Visual assessment is not always adequate, but bioengineering tools, such as LDF, are instrumental.[8]

The intensity of the wheal-and-flare response as mediated by histamine is related to the local reactivity of the blood vessels and to their indirect dilatation via the axon reflex. Unlike

the wheal that results directly from the effect of histamine on the blood vessels, the flare response is neurogenic, and it was shown that levels of histamine are not increased within the flare.[9] When the response to the first histamine prick decayed to half of its peak value, an additional prick resulted in a greater reaction, suggesting additivity. Following the first prick, vasodilatation did not reach maximum and the blood vessels were capable of further dilation. The reaction over the irritated site, however, was greater than predicted from adding the irritant reaction to the reaction to histamine. This may suggest that mediators in the cascade of events following histamine introduction are more readily activated at irritated skin. As histamine was intradermally administered, the first peak did not result from barrier disruption. It will be interesting to ascertain whether subclinical irritative damage will also elicit a higher response to histamine, as such damage is clinically important, and may have important environmental health implications.[10]

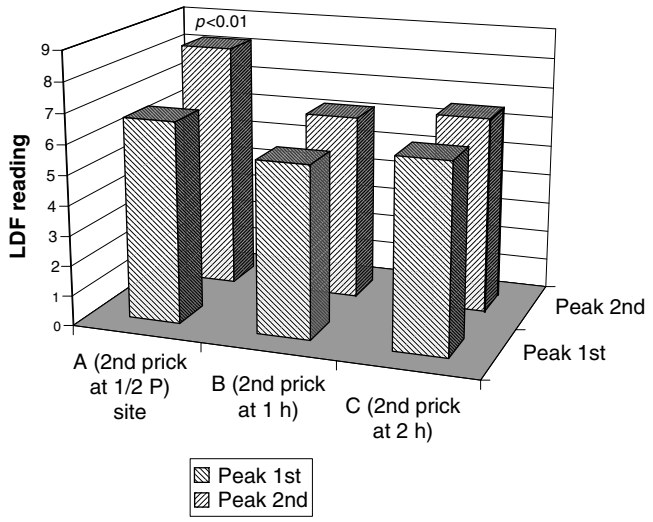


FIGURE 48.1 Laser Doppler flowmetry (LDF) readings at sites A, B, and C following the first (first row) and second (second row) histamine pricks for the 20 volunteers. (Reproduced from Tur, E., Tur, Z., Weltfriend, S., Schulze, K., and Maibach, H.I., *Exog. Dermatol*., 1, 39–44, 2002. With permission.)

Time parameters did not significantly differ among the various modes of histamine prick, including the first prick over the irritated site. The vasodilatory action of the histamine on the human skin blood vessels involves H_1 and H_2 receptors. Activation of either type of receptor can elicit maximum dilation, but the sensitivity to histamine, the duration of the effect and the mechanisms are different. H_1 receptors, which reside on endothelial cells, have a higher affinity for histamine and mediate a relatively rapid and short-lived response. By contrast, activation of the H_2 receptors, located on vascular smooth muscle cells, causes dilation that develops more slowly and is more sustained. In addition, the flare evoked by histamine is a manifestation of an axon reflex, as

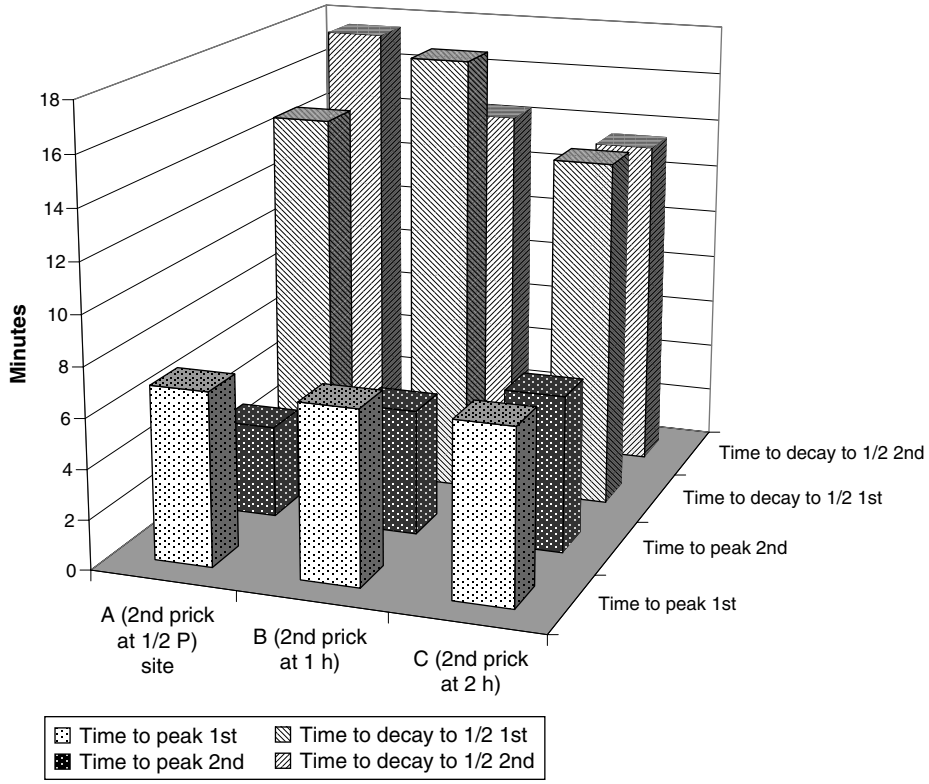


FIGURE 48.2 The times of the reaction over sites A, B, and C for the 20 volunteers. The first row depicts the time to peak of the first histamine prick; the second row: the second; the third row: the time to decay to half the maximum value of the first histamine prick; the fourth row: the second. (Reproduced from Tur, E., Tur, Z., Weltfriend, S., Schulze, K., and Maibach, H.I., *Exog. Dermatol*., 1, 39–44, 2002. With permission.)

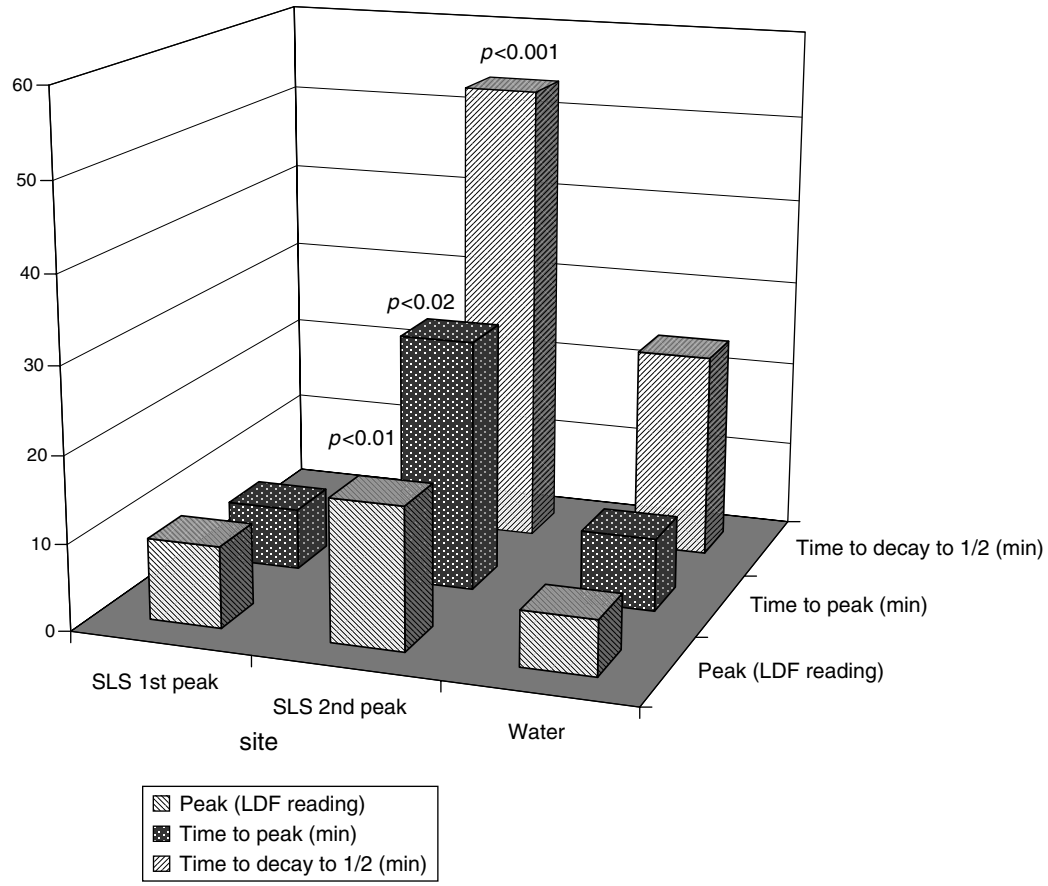


FIGURE 48.3 The parameters of the response to histamine prick following SLS and water 24 h patch for eight volunteers. The first row: the first and second peak values over the SLS patch, and the peak over the water patch. The second row: the time to reach these peak values. Third row: the time to decay to half the peak response over the SLS and water patch sites. (Reproduced from Tur, E., Tur, Z., Weltfriend, S., Schulze, K., and Maibach, H.I., *Exog. Dermatol*., 1, 39–44, 2002. With permission.)

it is blocked by local anesthesia. Following histamine injection, its blood levels rise in the wheal area but not the flare.[11]

The time similarities may suggest that the two types of histamine receptors were similarly activated at all modes of histamine provocation over intact skin. The reaction over irritated skin, however, started with the same activation as the other reactions, succeeded by an additional reaction. This later reaction may represent an H_2-receptor activation, which might have been affected by the prior exposure to SLS.

The reaction over irritated skin may also represent a reaction to an additional amount of histamine percutaneously delivered through the irritated skin. Following the prick, the additional amount absorbed through intact skin was negligible, whereas through irritated skin it might have been substantial, as exhibited by the additional later rise of the response and the prolonged time to decay. It has been shown that following topical application over intact skin on the back the time required to reach peak reaction is twice the time following intradermal administration, and the time to decay to half the response is one and a half times.[4] In the present study, the time required to reach the second peak over irritated skin was more than four times than following intradermal administration to intact skin, and the time to decay to half the response was more than double (Table 48.3). As penetration of topically applied substances through irritated skin should be greater and faster, the observed responses could not be explained by mere additional absorption, and therefore an enhancement of the response to histamine provocation is suggested.

Irritation may modulate cytokines:[12] inducible nitric oxide synthase was increased in irritant contact dermatitis,[13] and intravenous injection of histamine triggered a biphasic inflammatory cascade via initial extravasation process, and in the late phase was nitric oxide mediated.[14] Following SLS irritation, keratinocytes were activated and secreted soluble mediators, like vascular endothelial growth factor.[15] Such modulation may work in conjunction with histamine provocation to produce the enhanced response observed here.

Histamine elicits proinflammatory and immune-modulatory effects.[16] Not only irritation, but other external factors like triclosan and two other phenols were shown to affect the cascade reactions of inflammation elicited by histamine.[17] Occlusion by itself was shown to affect cytokines, decreasing the interleukin-1 alpha pool; it was suggested that these epidermal cytokines might play an important role in the signalling system.[18]

Tachyphylaxis is known to occur in human skin reactions to histamine.[19,20] In the present experimental setting,

it was not demonstrated. On the contrary, when the second prick was performed early enough (17.5 min, 2.1 S.E.), the reaction was higher. Histamine tachyphylaxis occurs in the respiratory smooth muscle, mediated via H_2 receptors, and is dependent on intact epithelium.[21] The rabbit celiac artery also showed tachyphylaxis to histamine-induced contractions, with involvement of H_2 receptors located on the endothelium.[22] Tachyphylaxis did not occur at the concentrations and the time points tested, but may occur at longer intervals, necessitating further experiments. Likewise, responses of rat spinal dorsal horn neurons to intracutaneous microinjection of histamine did not exhibit significant tachyphylaxis when readministered at 5 or 10 min intervals.[23,24]

Gathering data concerning the kinetics and duration of the pharmacological response at different time points of histamine administration would provide further information regarding the mechanisms underlying mediator-related processes. To explain the observed effect of irritation, the response to histamine prick over irritated skin should be studied with additional environmental irritants, whose properties and mechanisms of action differ from SLS.

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49 Specificity of Retinoid-Induced Irritation and Its Role in Clinical Efficacy

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49.1 INTRODUCTION

Most patients undergoing topical retinoid therapy experience "retinoid dermatitis," a type of irritant contact dermatitis characterized by erythema, scaling, dryness, burning, and pruritis (Webster et al., 2001; Leyden et al., 2001; Leyden and Grove, 2001). Although these symptoms are sometimes considered unpleasant, the relationship of this irritation to retinoid pharmacology and therapeutic benefit has been a topic of debate. Similarities between nonspecific irritant contact dermatitis and retinoid dermatitis raise questions about the specificity of retinoid-induced cutaneous changes. However, newer evidence suggests that some of these effects are, in fact, receptor-mediated and specific to topical retinoids.

49.2 RETINOID DERMATITIS

The term *retinoid* was traditionally used to describe natural and synthetic vitamin A (retinol) derivatives; however, this definition has been expanded to include all compounds that interact specifically with retinoid receptors (Leyden, 1998a). These compounds are used widely in clinical practice as topical therapy for the improvement of photoaged skin, acne, psoriasis (and other disorders of keratinization), as well as new applications to chemoprevention and wound healing. All-trans-retinoic acid (tretinoin or RA) is the major biologically active form of vitamin A and has been used successfully for over two decades (Bershad et al., 1999). Newer synthetic alternatives with retinoid receptor activity have been shown to be similarly effective; however, most are still associated with some degree of irritation.

In general, irritant contact dermatitis is characterized by nonimmunologic cutaneous changes caused by a wide array of irritants or abrasive agents (Denig et al., 1998). Clinical symptoms and signs vary significantly and may present acutely with erythema, edema, and vesicles. Chronic irritation may progress into severe dermatitis with scaling and desquamation after repeated exposure to irritants; cumulative exposure to low-grade irritants can cause increased damage to skin, if it is not allowed to heal adequately between exposures. Acute irritation may also elicit a delayed response, and some chemicals are known to cause acute irritant contact dermatitis 24 h or more after a single exposure to the irritant (Denig et al., 1998).

The clinical picture of retinoid dermatitis varies in intensity, and numerous patients who would benefit from this type of therapy discontinue treatment due to discomfort. Erythema, scaling, dryness, burning, and pruritus characterize the irritation (Webster et al., 2001; Leyden et al., 2001; Leyden and Grove, 2001). The majority of patients using topical tretinoin and other synthetic retinoids report that these symptoms subside after several weeks (Leyden and Grove, 2001; Appa, 1999; Webster et al., 2002; Lowe et al., 2004). However, one long-term clinical study showed that approximately half of the patients still experienced erythema, peeling, and burning after 24 weeks of treatment with 0.05% tretinoin (Olsen et al., 1997). It is known that susceptibility to retinoid-induced irritation varies among individuals. Interestingly, patients with skin types 1 and 2 experienced more irritation than those with 3 or 4 in a 14-day study of topical tazarotene 0.05% for psoriasis (Stucker et al., 2002).

Attempts to decrease irritation from topical tretinoin therapy include adding emollient cream to vehicle, an entrapped microsponge gel formulation, RA encapsulated in stratum corneum (SC) lipid liposomes, and a formulation that incorporates polyolprepolymer-2 to decrease penetration (Bershad et al., 1999; Leyden, 1998a, Quigley and Bucks, 1998; Contreras et al., 2005). In addition, synthetic receptor-specific retinoids, adapalene, tazarotene, seletinoid G, alitretinoin, MDI-301, have been designed as other clinically effective topical retinoids. Some of these (specifically adapalene, seletinoid G, and MDI-301) show promise for similar clinical efficacy with lower irritation potential. Other retinoids employed for therapeutic benefit are natural vitamin A (retinol), retinaldehyde and retinyl-palmitate, which appear inherently less irritating than RA (Fluhr et al., 1999; Kang et al., 1995); however, there is less evidence supporting their efficacy.

Emerging efforts to characterize mediators of retinoid dermatitis may allow for the identification of compounds that prevent or antagonize the inflammation. Kim et al. (2003) evaluated the changes in mRNA level of inflammation-related cytokines in mouse ear epidermis treated for 3 days with all-trans 2% retinol, as well as protein levels in cultures of human keratinocytes, melanocytes, and dermal fibroblasts incubated with 10 μM all-trans retinol or all-trans RA. They found the greatest increase in MCP-1 and IL-8 (Kim et al., 2003). Accordingly, they screened 10 potential anti-irritants prior to incubation to determine inhibitory effect on IL-8 and MCP-1 secretion. Of these, SC-glucan showed inhibition of retinol-induced irritation in the human patch test (Kim et al., 2003). Retinoic-acid receptor (RAR) antagonists, phenylcyclohexane, and phenylcyclohexadiene also inhibit cutaneous irritation induced by retinoids (Beard et al., 2001). Bz-432, a new benzodiazepine, inhibits retinoid-induced hyperplasia in cultured human keratinocytes (Varani et al., 2005). Soy extract and soy isoflavonones (genistein) also inhibit epidermal hyperplasia in cultured keratinocytes, while maintaining beneficial effects in dermal fibroblasts (Varani et al., 2004). However, it is not known to what extent these agents interfere with the therapeutic effects of topical retinoids.

49.3 SPECIFICITY OF RETINOID DERMATITIS

Retinoids have been compared to well-studied irritants such as sodium lauryl sulfate (SLS) to examine the specificity of retinoid-induced irritation attempting to answer two questions: (1) Is retinoid dermatitis a nonspecific irritant effect of topical retinoid therapy or is the irritation directly related through receptor signaling to retinoid influence on cell function? (2) Is this irritation responsible for the therapeutic benefit attributed to topical retinoids? In other words, would long-term application of another irritant or abrasive agent give the same clinical results? Research suggests that certain retinoid-induced cutaneous changes are retinoid-specific, while other histological and clinical effects of topical therapy are mimicked by application of other irritants and abrasive agents (Marks et al., 1990; Fisher et al., 1991; Landecker et al., 2001).

Criticism that the irritation induced by tretinoin was responsible for its reparative effects led to studies comparing topical tretinoin with other irritants and abrasive agents. It was proposed that therapeutic actions may not be specific to retinoids, and could be achieved by the application of other nonspecific irritants. In fact, histological changes induced by nonspecific irritants are strikingly similar to RA. Fisher et al. (1991) observed the cutaneous changes after 4-day patch test with the irritant SLS or 0.1% RA were indistinguishable. Within 2–5 days, an erythematous scaling reaction occurs accompanied by SC compaction, epidermal thickening, hyperplasia, increased mitotic figures, spongiosis, and inflammation (Fisher et al., 1991). Similar histological alterations are also documented when 0.05% tretinoin is compared to the irritant/peeling agent glycolic acid (Landecker et al., 2001), or an aluminum oxide in soap paste (Marks et al., 1990). It is also known that retinoids have a detergent-like effect on cellular membranes (Meeks et al., 1981), which may contribute to irritation potential.

Interestingly, subsequent studies demonstrating differences in the timing of onset of these effects suggest that they are induced by different pharmacotoxicological mechanisms. In a 24-h occlusive patch-test assay, SLS immediately disrupts the barrier function of the SC through direct cytotoxic effects on keratinocytes; maximal trans epidermal water loss (TEWL) is seen within the first 1–2 days after application (Effendy et al., 1995, 1996; Ale et al., 1997). This drying effect decreases progressively, but may last for more than 10 days after a single occlusive application before returning to baseline values (Effendy et al., 1996; Ale et al., 1997). In contrast, the TEWL increase after RA application is delayed and less significant than SLS. Moreover, this effect was followed by an overall increase in SC hydration as measured by capacitance. Other studies confirm that topical all-trans RA is noncorrosive when compared to SLS (Fullerton and Serup, 1997). This may be secondary to RAs induction of increased epidermal cell proliferation and scaling, and to glycoconjugates accumulating in the epidermis (Effendy et al., 1996; Ale et al., 1997). However, SLS may also increase SC turnover, although to a lesser extent, when applied cumulatively (Effendy et al., 1996). Glycolic acid, another irritant, is also capable of increasing SC proliferation, but with less hyperplasia and scaling than seen in RA-treated human skin (Effendy et al., 1995).

49.4 ROLE OF RETINOID RECEPTOR SIGNALING

Retinoid action in the skin and the irritation correlate to RA receptor-γ (RAR-γ) activation (Chen et al., 1995). Of the three RARs (γ, α, and β), RAR-γ is responsible for 90% of RAR in human skin, signals as a heterodimer with RXR-α, is mandatory for receptor function (Fisher and Voorhees, 1996; Feng et al., 1997; Thacher et al., 1997), and binds RA with the greatest affinity (Fisher and Voorhees, 1996). Therefore, it is most likely responsible for the majority of retinoid-induced transactivation of target genes (Fisher and Voorhees, 1996). Eleven retinoids were shown to prefer RAR-γ as determined

by transcriptional activity (Chen et al., 1995). These compounds were then tested for clinical efficacy against acne and irritation potential in the rhino mouse utriculi reduction assay and in the rabbit irritation model respectively. Both efficacy and irritation correlated strongly and positively with each other and with RAR-γ transactivation activity. Consequently, numerous receptor-specific synthetic retinoids have been introduced in hopes of identifying effective, nonirritating retinoids. RAR-α and RAR-β agonists may indeed have lower irritation potential (Standeven et al., 1997; Vuligonda et al., 1999); however, the relationship among receptor specificity, irritation, and clinical efficacy is not clear. Synthetic nonirritating retinoids, specifically MDI-301 and seletinoid G, appear to induce retinoid-specific changes in skin without clinically appreciable irritation (Varani et al., 2003; Kim et al., 2005). It is likely that such a formulation will soon be commercially available; however, there is limited data comparing their efficacy to other established topical retinoids.

Examination of the relationship of RAR–RXR heterodimer signaling and the clinical and histological effects of topical retinoid treatment has provided insight into the mechanism of retinoid-induced irritation. When the RXR partner of the RAR–RXR heterodimer is targeted by selective retinoids, there was no significant irritation or hyperplasia; RXR is probably a silent partner in heterodimer signaling (Thacher et al., 1997). The direct association between RAR signaling and cutaneous response was established with a genetically engineered mouse model shown to overexpress dnRXR-α, and sequester the majority of functional RAR-γ, leading to a functional deficiency of RARs (Feng et al., 1997). Topical tretinoin failed to induce the hyperplasia and desquamation response in the epidermis of these mice (Feng et al., 1997). Therefore, RAR signaling induces genes that cause this hyperproliferative response.

We now know that epidermal hyperplasia is mediated by retinoid-induced upregulation of heparin-binding epidermal growth factor receptor (EGFR) ligands (Rittie et al., 2006; Kimura et al., 2005; Varani et al., 2001; Yoshimura et al., 2003). After inhibition of EGFRs, topical retinoids fail to induce epidermal hyperplasia. However, clinical irritation may be, at least in part, separable from these histologic effects (Rittie et al., 2006). All-trans retinol is also known to signal through RARs and produce similar histological effects to tretinoin, but with no clinical erythema (Kang et al., 1995; Kang and Voorhees, 1998). Moreover, the compound MDI-301 (synthesized from 9-*cis*-retinoic acid by replacing the carboxylic acid with an ester linkage) induces epidermal hyperplasia in murine skin with no clinically evident erythema or scaling (Varani et al., 2003).

Perhaps the most convincing demonstration of RA's unique pharmacological properties is its ability to stimulate collagen synthesis in photoaged skin. Kligman et al. (1996) compared tretinoin with other irritants and peeling agents: glycolic acid, benzalokium chloride, SLS, croton oil, and vehicle. Tretinoin-treated hairless mouse skin had increased amounts of type-III procollagen and collagen and showed a repair zone of new collagen twice the depth of vehicle or irritant-treated sites (Kligman et al., 1996). After 4–12 months of treatment with tretinoin, photoaged human skin shows increased collagen synthesis in the papillary dermis (Kligman et al., 1996; Griffiths, 1993; Woodley et al., 1990). Also, aberrant elastic fibers in the dermis of photoaged forearm skin were reorganized after a 4-day occluded patch application of 0.025% tretinoin (Watson et al., 2001). After treatment with SLS, no obvious change in this parameter was observed (Watson et al., 2001). These dermal effects of RA suggest a specific mechanism for RA in repair of solar-induced damage that does not depend on irritation, but on collagen synthesis and elastic fiber reorganization.

Clearly, RA exerts its pharmacological and irritant effects through mechanisms distinct from other nonspecific irritants. This statement is supported by evidence that RA exerts its specific actions through receptor-mediated cell signaling and induction of genes containing RAREs (retinoic-acid response elements).

49.5 ROLE OF IRRITATION IN CLINICAL EFFICACY

49.5.1 PHOTOAGING

The notion that SLS and other irritants/abrasives may improve the appearance of photodamaged skin is reasonable; however, it is unlikely that irritation alone is responsible for tretinoin-induced repair of photoaging. Clinically, irritant dermatitis is not necessary to demonstrate effect. Early studies comparing 0.1 and 0.025% tretinoin found a threefold greater incidence of erythema and scaling with 0.1% tretinoin with no significant difference in clinical efficacy for photoaging between the two concentrations (Griffiths et al., 1995). Alitretinoin gel (0.1%) may also prove effective in repairing photodamaged skin, although there are no studies comparing its irritation potential to other retinoids (Baumann et al., 2005). Recent data suggest that tazarotene, a more irritating retinoid, may be superior to tretinoin for the treatment of photoaging (Lowe et al., 2004). However, Kim et al. (2005) designed seletinoid G, a synthetic RAR-agonist that is truly nonirritating after a 4-day occlusive application. It is indeed capable of repairing photodamaged human skin *in vivo* and supports the concept that retinoid dermatitis is not required for the treatment of photoaging.

Retinoid-induced repair of photodamage is thought to occur through a variety of unique mechanisms. Specific repair occurs in the dermis—partly through new collagen synthesis, replacement of sparse microfibrillar apparatus, and reorganization of aberrant elastic fibers in the papillary dermis (Kligman et al., 1996; Griffiths, 1993; Woodley et al., 1990; Watson et al., 2001). Tretinoin-treated hairless mouse skin had increased amounts of type-III procollagen and collagen and showed a repair zone of new collagen twice the depth of vehicle or irritant-treated sites (Kligman et al., 1996). After 4–12 months of treatment with tretinoin, photoaged human skin shows increased collagen synthesis in the papillary dermis (Kligman et al., 1996; Griffiths, 1993; Woodley et al., 1990).

Also, aberrant elastic fibers in the dermis of photoaged forearm skin were reorganized after a 4-day occluded patch application of 0.025% tretinoin (Watson et al., 2001). Subsequent studies on topical retinoid therapy for photaging have supported these observations that retinoids act by several specific mechanisms to repair and prevent UV-induced damage (Olsen et al., 1997; Landecker et al., 2001; Thorne et al., 1996). The novel synthetic retinoid, seletinoid G, has been shown to increase expression of type-I procollagen, tropoelastin, and fibrillin-1 (Kim et al., 2005).

The pharmacological activity of tretinoin also uniquely influences other aspects of cell biology related to its pharmacological activity. RA has growth-promoting properties separable from its cytotoxic effects, and it supports growth of human epidermal keratinocytes in organ culture (Varani et al., 1993). Specific induction of RARE-containing genes expresses markers for RA-specific action in the skin, inducing the cellular RA-binding protein II gene and protein (Elder et al., 1993a and b) and the RA-inducible skin-specific gene/psoriasin; both markers preceded erythema in response to topical tretinoin (Zouboulis et al., 1996). Fibrillin-1 is increased in the dermis of photoaged skin after long-term RA therapy and may serve as a reporter agent for photodamage repair (Watson et al., 2001). Although, SLS increases fibrillin-1 to a significantly lesser extent than RA, it is not associated with reorganization of elastic fibers in the papillary dermis or increased collagen synthesis (Kligman et al., 1996; Watson et al., 2001).

In addition to repairing existing solar-induced damage, RA and seletinoid G have both been shown to prevent future damage by blocking UVB-induced activation of collagen degrading matrix metalloproteinases, a crucial mechanism of photoaging in skin (Fisher et al., 1996, 1998; Varani et al., 2000; Brennan et al., 2003; Kim et al., 2005). Collagen fragments may then inhibit fibroblast production of procollagen, thus perpetuating a cycle of damage (Fligiel et al., 2003). After topical application, retinyl esters concentrate in the epidermis and directly absorb UVB radiation (Antille et al., 2003; Sorg et al., 2005). In animal models, topical retinoids also upregulate CD44, a cell surface receptor for hyaluronate, which maintains its homeostasis in mouse skin, thereby increasing hyaluronate synthases (Kaya et al., 2005) UVA and UVB decrease the expression of these molecules, an effect that is antagonized by topical retinoids when they are applied prior to exposure (Calikoglu et al., 2006). Thus, the specific properties and pharmacological actions of topical retinoids contribute to their ability to repair existing photodamage and prevent new damage from occurring. This therapeutic benefit appears to be independent of the irritant effects of these molecules.

49.5.2 Acne

Topical tretinoin therapy for acne vulgaris improves abnormal desquamation of the follicular epithelium, reduces cellular cohesion, and helps minimize the accumulation of excessive sebum and *Propionibacterium acnes* proliferation, thus inhibiting microcomedo formation (Leyden, 1998; Shalita, 2001). Recently, topical retinoids have been shown to be effective for inflammatory lesions (Leyden et al., 2005, 2006; Thiboutot et al., 2006). Topical retinoids are also slightly bactericidal (Shalita, 2001). As mentioned earlier, this therapy is associated with retinoid dermatitis; it is not clear if a nonirritating compound may have similar efficacy to more irritating formulations, although, newer synthetic retinoid compounds have been compared to tretinoin to assess irritation potential. Since these effects occur mostly in the epidermis, some degree of irritation has been associated with efficacy in the treatment of acne. MDI-301 was shown to reduce utriculi in murine skin without evidence of irritation and may prove effective for acne therapy in human skin (Varani et al., 2003). However, commercially available retinoids used for acne are still associated with some degree of scaling and erythema. Tazarotene is synthetic retinoid shown to be highly effective, and it has irritation potential similar to or greater than tretinoin (Webster et al., 2001; Leyden et al., 2001; Leyden and Grove, 2001). Several studies suggest that the napthoic acid derivative adapalene had less irritation potential (Verschoore et al., 1997; Griffiths et al., 1998; Galvin et al., 1998; Clucas et al., 1997; Alirezai et al., 1996) and equivalent efficacy against acne (Shalita et al., 1996; Cunliffe et al., 1998; Ioannides et al., 2002), although some argue that greater irritation is directly related to potency as acne treatment (Bershad et al., 1999). Interestingly, in a 4-day patch test on human skin, adapalene does not induce the epidermal hyperplasia, spongiosis, and erythema characteristic of RA-treated skin, although it does induce CRABP-II expression (Griffiths et al., 1993), which is thought to be a marker for retinoic acid action in human skin. It also leads to epidermal hyperplasia and comedolysis in the rhino mouse model (Griffiths et al., 1993). Other possible explanations for reduced irritation with adapalene treatment include that it is relatively RAR-β selective (Verschoore et al., 1997), or that it may be targeted to follicles (Leyden, 1998a and b). Nevertheless, there is evidence, which contradicts these findings (Leyden et al., 2001; Leyden and Grove, 2001; Shalita, 2001). Some advocate the concept that adapalene is comparable to tretinoin in both efficacy and irritation capacity, and that irritation among different retinoids is more related to individual susceptibility than to inherent irritant capacity of the different compounds (Leyden et al., 2001; Leyden and Grove, 2001; Shalita, 2001). Since adapalene does bind RAR-γ, the latter trials agree with our current knowledge of RAR-γ mediated retinoid-induced cutaneous effects, as well as our understanding of the mechanism by which retinoids improve acne. The problem with correlating these histological effects with irritation and clinical efficacy in humans is that these parameters are more difficult to measure and quantify.

49.6 CONCLUSIONS

Retinoid dermatitis is distinct from nonspecific irritant contact dermatitis in that the former is at least partly a receptor-mediated process rather than the result of direct cytotoxicity.

RAR signaling induces retinoid effects including hyperproliferation, organization, desquamation, and erythema. In addition, topical retinoids induce gene expression leading to new collagen synthesis and reorganization of the dermal connective tissue, and directly absorb UVB.

Irritation potential may be related to individual patient susceptibility or among differences among topical retinoids. In addition, therapeutic effects may, at least in part, be separable from irritant effects. Still, there remains controversy over whether newer synthetic retinoids can demonstrate equal clinical efficacy without significant irritation. There is no question that retinoid dermatitis is partially related to its pharmacological effects, although, the mechanism of retinoid actions on the skin is still incompletely understood. Taken together, we still lack precise quantification and qualification of the difference between the clinical and irritant effects of these molecules.

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50 Topical Corticosteroids in the Treatment of Irritant Dermatitis: Do They Work?

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50.1 INTRODUCTION

Corticosteroids are currently employed in the treatment of various dermatological disorders, including atopic eczema, psoriasis, allergic contact dermatitis, and irritant contact dermatitis (ICD). Oral, intralesional, and topical formulations of corticosteroids exist, though topical ones are preferred in the treatment of dermatologic conditions. Topical formulations induce a local response and have minimal systemic effects. While topical corticoids are effective in treating ICD in animals, their clinical efficacy in humans provides conflicting results (Peets and Maibach, 1995). This chapter reviews topical corticoid use in the treatment of ICD in humans.

50.2 BIOENGINEERING MEASUREMENTS

Objective assessment of topical corticosteroid efficacy in treating ICD requires both visual and quantitative measures. The use of noninvasive bioengineering equipment, including the transepidermal water loss meter, the chromameter, and the laser Doppler flowmeter (LDF), has aided in this endeavor. They allow quantification of skin damage that otherwise may be clinically undetectable.

50.3 CLINICAL INVESTIGATIONS

Only a handful of studies exist that evaluate the efficacy of topical corticosteroids in treating ICD in humans using controlled quantitative experimentation (see Table 50.1). Van der Valk and Maibach (1989) studied the effects of several topical corticosteroids on ICD in man, including clobetasol-17-dipropionate, hydrocortisone 1%, and triamcinolone acetonide 0.1%. Sodium lauryl sulfate (SLS; 0.36%) was used to induce a uniform dermatitis on the volar forearms of 17 otherwise healthy subjects. Application for 45 min of occlusive patches twice daily for a total of 3 weeks produced a cumulative irritant dermatitis. Immediately upon removal of the first patch of the day, 0.088 g/cm² of corticoids were openly applied onto the irritated skin. Utilizing both a visual grading scale for erythema and transepidermal water loss (TEWL) to assess the irritation, the study found no significant effect of corticosteroid application when compared with vehicle-treated skin. In fact, TEWL increased slightly upon clobetasol application.

Le et al. (1997) studied the effects of corticosteroids on cumulative ICD using a similar design as Van der Valk et al. ICD was induced with a solution of 0.2% SLS applied for 4 h once daily for 5 consecutive days. Patches were applied

TABLE 50.1
Human *In Vivo* Clinical Trials of Corticosteroids in the Treatment of Irritant Dermatitis

Experiment	Corticosteroid(s) Utilized	Efficacy
Van der Valk and Maibach, 1989	Clobetasol-17-dipropionate (CBD) cream Hydrocortisone in CBD Hydrocortisone in petrolatum Triamcinolone acetonide in petrolatum Vehicles of each	No effect with any corticosteroid when compared with vehicle-treated skin
Ramsing and Agner, 1995	Betamethasone-17-valerate cream and vehicle	Slightly significant improvement on day 7 when compared with vehicle-treated skin; no effect on days 1–6
Berardesca et al., 1995	Methyl prednisolone aceponate (MPA) in cream 5% Linoleic acid cream (LAC) Placebo base cream (PBC)	7% Mean reduction with LAC on day 4 and MPA on days 3 and 4 when compared with nonirritated skin. No effect on other days or when compared with irritated skin. No comparison of LAC or MPA with vehicle-treated skin
Le et al., 1997	Triamcinolone acetonide 0.05% cream and its vehicle	No significant effect was observed
Levin and Maibach, 2001 (irritated with SLS)	Betamethasone valerate (BMV) ointment Hydrocortisone ointment Petrolatum vehicle	No significant effect was observed
Levin and Maibach, 2001 (irritated with NAA)	Betamethasone valerate ointment Hydrocortisone ointment Petrolatum vehicle	BMV was minimally effective on day 8. Petrolatum vehicle reduced LDF on day 3. No other effects were observed

onto the back skin on 24 healthy volunteers. The efficacy of triamcinolone acetonide (0.05%) cream and its vehicle was tested. 0.094 g/cm^2 of the creams were openly applied once daily for 7 days. Both visual grading and TEWL were assessed on days 1–7 and on days 10 and 14. Neither TEWL nor visual grading was significantly affected by corticosteroid application when compared with vehicle control.

Ramsing and Agner (1995) studied the effect of corticosteroids in treating acute irritant dermatitis. Twenty-four-hour patch application of 0.5% SLS induced the dermatitis on upper arm of 16 hand eczema patients. Upon patch removal, either betamethasone-17-valarate ointment or its vehicle was applied onto the irritated skin. Open application of corticosteroids occurred twice daily for 7 days. TEWL, spectrophotometry, and visual grading were used to quantify results immediately upon patch removal and on days 4 and 7. In contrast to Van der Valk's findings, Ramsing found that the corticosteroids reduced both TEWL and erythema on day 7 when compared with contralateral vehicle-treated skin. Interestingly, no significant change in TEWL was observed during the first 6 days of treatment or when comparing treated and vehicle-treated skin on the same arm. Additionally, only a 10% median TEWL reduction was observed. The clinical relevance of this slight improvement is unclear.

Utilizing a similar methodology to the Ramsing study, Berardesca et al. (1995) induced an acute irritation with 24-h patch application of 5% SLS onto the volar forearms of nine healthy volunteers. The efficacy of corticosteroids 0.1% budesonide in cream (BUD), 0.025% methyl prednisolone aceponate in cream (MPA), 5% linoleic acid cream (LAC), and placebo base cream (BAC) were studied. Corticosteroids were applied via open application. MPA was applied once daily, while BUD, LAC, and BAC were applied twice daily for a total of 4 days. In seeming support of the study by Ramsing et al., this study found a significant decrease in TEWL with the LAC treatment on day 4 and with the MPA treatment on both days 3 and 4 when compared with nonirritated skin. There was only a 7% mean TEWL reduction observed, which also questions the clinical relevance of these findings. Though the efficacy of placebo was tested, a comparison between treated and placebo-treated skin was not recorded. LAC and BAC were not significantly different from irritated skin.

In two experiments by Levin et al. (2001), corticosteroid efficacy was tested using either SLS or nonanoic acid (NAA)-induced irritation. In one study, an acute ICD was induced via open application of 10% SLS onto the dorsal hands of six healthy volunteers using a previously described hand-washing assay (Charbonnier et al., 2000). The SLS solution was rubbed into the volunteers' hands every hour for a total of five daily hand washes in 1 day. Immediately following the final washing, 18 mg/cm^2 of betamethasone valerate, hydrocortisone, and petrolatum vehicle were openly applied. Corticosteroids were applied once on day 1 and twice daily for an additional 4 days. TEWL, chromametry, and visual scorings of both erythema and dryness assessed results. Squamometry (Ale et al., 1996; Pierard

et al., 1992), a method involving analysis of staining and analysis of a stratum corneum tape stripping, was also performed. The study did not observe any significant difference between corticosteroid-treated and either vehicle-treated or untreated sites.

In the second study, NAA was utilized to induce an acute irritation (Levin and Maibach, 2001). Twenty-four-hour patch application of both 90 and 60% NAA dissolved in isopropanol induced the ICD on the volar forearms of 11 healthy volunteers. Betamethasone valerate, hydrocortisone, and petrolatum (vehicle) were applied via patch application to the irritated sites twice daily for 4 consecutive days. TEWL, chromametry, LDF, and visual scoring of both erythema and dryness were utilized to quantify results. The study found betamethasone-17-valerate minimally effective in treating 90% NAA-induced acute irritant dermatitis when compared with untreated control, as quantified by TEWL on day 8 of experimentation. Chromametric values of betamethasone were also significantly lower than hydrocortisone on day 8 and approached significance when compared with untreated control. Interestingly, petrolatum had a significant effect in reducing LDF values when compared with untreated control on day 3. Given that significant differences were only observed on 1 day and were not a general trend throughout the experimentation, the clinical value of these results is questionable.

50.4 DISCUSSION

50.4.1 Skin Site

In the reviewed studies, ICD was induced on the dorsal hands, volar forearm, flexor upper arm, and back skin of healthy volunteers. In everyday life, irritant dermatitis is most often localized to the hands. However, experimentation suggests that the forearm is an appropriate model for assessing efficacy of treatment on irritant dermatitis (Charbonnier et al., 2000; Held and Agner, 1999; Paye et al., 1999). In contrast, skin barrier properties on the skin of the back may drastically differ from that of the hand, due to the significantly increased thickness of the stratum corneum on the back (Held and Agner, 1999). Therefore, comparison between irritation of the back and of the hands should be interpreted with caution. Of note, in a recent study, irritation of the human scalp as compared to the upper back and volar forearm demonstrated that the back was most sensitive to SLS challenge (Zhai et al., 2004). This small study supports use of the upper back as a model for irritant dermatitis. To date, there are no studies to evaluate whether the upper arm is an appropriate model for SLS irritation or not.

50.4.2 Lipophilic versus Hydrophilic

In the reviewed experimentation, the surfactants SLS or NAA were used to induce ICD. Both SLS and NAA have the capacity to lightly damage the skin and are not sensitizers or carcinogens; nor do they cause excessive discomfort to human volunteers (Wahlberg and Maibach, 1980). The hydrophilic SLS and lipophilic NAA may exhibit differing physicochemical characteristics and thereby irritate the skin utilizing different mechanisms of action. It is known, for example, that skin barrier function is only minimally affected by NAA application (Agner and Serup, 1989), while greatly damaged by SLS (Miyazawa et al. 1984). Topical corticosteroid application may provide more benefit to NAA-damaged skin than SLS-induced irritation.

50.4.3 Patch versus Open Application

Another variation among the aforementioned studies includes the method by which the surfactant was applied to the skin. Most of the studies utilized a patch application of irritant. However, ICD is often induced through open, repeated exposure to detergents and other chemical irritants. Open application of surfactant, therefore, would mimic a more realistic clinical scenario (Lee and Maibach, 1994; Tupker, 1997; Wilhelm et al., 1990). In addition, it seems likely that ICD initially damages the superficial stratum corneum skin layer. With occlusive application of surfactant, deeper layers of skin may be initially affected. Open application and patch testing may investigate different aspects of skin barrier function.

50.4.4 Acute versus Cumulative

Excluding severe chemical toxicity, irritant dermatitis most often results from cumulative exposure to a minimally irritating chemical. Ramsing and Agner (1995), Berardesca et al. (1995), and Levin and Maibach (2001) studied the effects of corticosteroids in treating acutely induced dermatitis. Single skin challenge certainly reflects the transient susceptibility of the skin to the particular irritant, but it does not investigate repair mechanisms to cumulative irritation (Grunewald et al., 1995; Lammintausta et al., 1998). The repetitive dosing methodology utilized by Van der Valk and Maibach (1989) and Le et al. (1997) better satisfies this criterion.

Most of the clinical studies tested the effect of corticosteroid following removal of the irritant. While Ramsing, Berardesca, and Levin found a slight improvement with the corticosteroids, Le found no improvement. The effect of corticosteroids while maintaining the causative ICD, as investigated by Van der Valk, suggest that corticosteroids are not effective when the source of irritation is not eliminated.

50.4.5 Ethnicity

A recent study by Astner et al. (2006) suggests that African-American skin may be more resistant to irritant dermatitis as compared to Caucasian skin. The clinical irritant studies presented in this chapter were all performed on Caucasian skin. Therefore, caution is advised when applying the study results to different skin colors or types.

50.4.6 Other Experimental Conditions

Experimental conditions such as the type of chamber utilized (Nicholson and Willis, 1999), the temperature of application

TABLE 50.2
Experimental Variables in Corticosteroid Treatment of Irritant Dermatitis

Irritant characteristics, such as molecular weight, charge, solubility
Anatomic skin site
Occlusion versus open application
Skin temperature
Ethnicity
Age
Airflow
Acute versus chronic irritation

(Ohlenschlarger et al., 1996), and the concentration of surfactant (Agner and Serup, 1990; Sugar et al., 1999) may also affect study results. In addition, recent experimentation suggests that warm airflow in the testing room (Fluhr et al., 2005) may exacerbate the irritant response, while cold has been noted to prevent irritation (Table 50.2).

Based on the evidence so far, corticosteroid formulations do not appear beneficial in treating lipophilic- or hydrophilic-induced ICD. If corticosteroids are not effective in an experimentally controlled environment, it seems unlikely that they will prove beneficial in a clinical setting.

50.5 ADVERSE EFFECTS

Even if corticosteroids are not proven to be effective as a treatment, physicians may be tempted to prescribe these agents for their placebo effect. Therefore, it is prudent to discuss some of the adverse effects associated with topical corticosteroid use.

50.5.1 Systemic Effects

Many of the associated serious effects have resulted from high-potency steroids. Fortunately, local effects are significantly more common than their systemic counterparts. Cushing's syndrome, a disorder in which there is an overproduction of cortisol, has been reported among topical corticosteroid users (Keipert and Kelly, 1971; Maldonado, 1982; Staughton and August, 1975; Siklar et al., 2004). Fatalities due to corticosteroid-induced Cushing's syndrome have rarely been observed (Nathan and Rose, 1979). Renal and hepatic disease patients are at higher risk (Fiewel et al., 1969). Symptoms and signs include upper body obesity, osteoporosis, muscle atrophy, hypertension, hyperlipidemia, and hyperglycemia.

Topical corticosteroids have also been implicated in laboratory-induced adrenal suppression, as indicated by one or more adrenocortical tests, including reduced morning plasma cortisol levels and decreased plasma cortisol response to the ACTH test (Gilbertson et al., 1998; Lawlor and Ramabala, 1984; Ortega et al., 1975). There have only been a few reported cases of clinical adrenal suppression due to topical corticosteroid use (Altura, 1966; Sneddon, 1970). While the systemic risks should be appreciated, the frequency and severity of local effects may not be overlooked. There are more than 20 different local reactions to corticosteroids (see Table 50.3) (Kligman, 1979). Often, the local effects can be devastating for the patient and may result in a discontinuation of use. The most common adverse effect, atrophy, was observed by Epstein et al. (1963). Atrophic skin appears thin and often presents with telengiectasiae and striae (Stevanovic et al., 1977). Thinning of the epidermis and regression of the papillary dermis are evident upon histological inspection (Chernosky and Knox, 1964).

TABLE 50.3
Adverse Effects of Topical Corticosteroids

Effects	
Systemic	**Local**
Cushing's syndrome	Dermal atrophy, striae
Adrenal suppression	Telangectasiae, purpura
Osteoporosis	Rosacea, perioral dermatitis
Muscle atrophy, myopathy	Acne, folliculitis
Growth retardation	Hypopigmentation
Cataracts, glaucoma	Skin infections
Hypertension	
Hyperlipidemia	
Hyperglycemia	
Obesity	
Immunosuppression	
Infections	
Hirsutism	
Psychiatric problems	

50.5.2 Local Effects

Severe dermal atrophy may lead to fragility of blood vessels that may explode upon trivial trauma (Maibach, 1979). The result is purpuric lesions, and eventually stellate pseudoscars. Scarring is most frequent on the extremities. In extreme cases, ulceration may result secondary to the purpuric lesions (Kligman, 1979).

Corticosteroid-induced rosacea and acne are two other relatively common local effects from glucocorticoid application. Patients with corticosteroid-induced rosacea are with intermittent papulopustules on the face. More potent corticosteroids are given to the patient to treat the facial lesions, which may initially improve the lesions. However, the eventual result is a more severe rebound of rosacea (Sneddon, 1969). Physicians should recognize the facial lesions as rosacea and encourage withdrawal of corticosteroid use. A specific form of rosacea, namely perioral dermatitis, is especially common among corticosteroid users. Perioral dermatitis describes the formation of follicular papules and pustules with a circumoral distribution. The skin adjacent to the vermillion border is spared. In general, the fluorinated steroids are most often responsible for the dermatitis (Sneddon, 1976). Corticosteroid-induced acne is a distinctive monomorphic follicular eruption (Kligman, 1988; Nikolowski, 1976). The acnegenic effect may

result from a degeneration of the follicular epithelium and an extrusion of the follicular contents (Kaidbey and Kligman, 1974; Plewig and Kligman, 1973). Preexisting active acne may initially be suppressed by corticosteroid application. Shortly thereafter, however, new lesions appear further aggravating the acne.

50.6 CONCLUSION

Taken together, the risk–benefit ratio of using topical corticosteroids for the treatment of irritant dermatitis remains unclear. The risk is well established. What is uncertain is corticoids' clinical benefit in experimental models of irritant dermatitis. Until a clear effect with topical corticosteroids is observed, other treatment options including prevention, cool compresses, and UV therapy should also be considered. However, in clinical practice, as corticosteroids remain first-line treatment of endogenous eczema, it follows that the clinician will also prescribe them for irritant dermatitis.

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51 Tests for Sensitive Skin

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51.1 INTRODUCTION

Sensitive skin is a condition of subjective cutaneous hyperreactivity to environmental factors or topically applied products. The skin of subjects experiencing this condition reacts more easily to cosmetics, soaps, and sun screens and often enhance worsening after exposure to dry and cold climates.

Sensitive skin and subjective irritation are widespread since the use of cosmetics is increasing in economically advantage countries.

The frequent use of preservatives, perfumes, emulsifiers, and plant extracts in fact enhance risk of adverse local reactions.

Signs of discomfort as itching, burning, stinging, and a tight sensation are commonly present, associated or not with erythema and scaling.

Generally, substances that are not commonly considered irritants are involved in this abnormal response. They include many ingredients of cosmetics such as dimethyl sulfoxide, benzoyl peroxide preparations, salycilic acid, propylene glycol, amyldimethylaminobenzoic acid, and 2-ethoxyethyl methoxycinnamate [1]. The unpleasant sensations appear to be associated with the stimulation of cutaneous nerve endings specialized in pain transmission, called *nociceptors.*

Some authors [2] hypothesized a correlation between sensitive skin and constitutional anomalies or other triggering factors such as occupational skin diseases or chronic exposure to irritants; others [3] supported that no constitutional factors play a role in the pathogenesis of sensitive skin, though the presence of dermatitis demonstrates a general increase in skin reactivity to primary irritants lasting months.

In different epidemiological surveys, the correlation between sensitive skin with sex, race, skin type, and age has been studied. No sex-related significant differences have been found in the reaction pattern.

Some authors [4–6] documented a higher reactivity to irritants mostly in females, some others noted that male subjects were significantly more reactive than female [7] but other experimental studies did not confirm these observations [8,9].

Conflicting data were also reported on skin sensitivity among races: although blacks seem to be less reactive and Asians more reactive than Caucasians, data rarely reach statistical significance [10]; recently, Arakami found significant subjective-sensory differences between Asian and Caucasian women but no differences after sodium lauryl sulfate (SLS) testing concluding that stronger sensations in Asians can reflect a different cultural behavior rather than measurable differences in skin physiology [11].

Studying the correlation between skin reactivity and skin type subjects with skin type I were found to be more prone to develop sensitive skin [12]; most common "stingers" were reported to be light-complexioned persons of celtic ancestry who were sunburned easily and tanned poorly [13].

Moreover, skin reactivity tends to decrease with age: by testing croton oil, cationic and anionic surfactants, weak acids, and solvents, less severe skin reactions were observed in older subjects [14]. Robinson [15], by testing sodium dodecyl sulfate, decanol, octanoic acid, and acetic acid, confirmed this lower reactivity in the older age cluster of subjects.

Aged skin seems to have a reduced inflammatoy response either to irritants or to irritation induced by UV light [16,17]. However, skin reactivity of women at the beginning of the menopause is increased, suggesting a role of estrogen deficiency on the observed impairment of skin barrier function [18].

51.2 TESTS FOR SENSITIVE SKIN

51.2.1 Clinical Parameters

It is difficult to find accurate parameters for categorizing skin as sensitive or nonsensitive; this condition often lacks visible, physical, or histologically measurable signs. Subjects with subjective irritation tend to have a less hydrated, less supple, more erythematous, and more teleangiectatic skin, compared to the normal population. In particular, significant differences were found for erythema and hydration/dryness [19]. Tests for sensitive skin are generally based on the report of sensation induced by topically applied chemicals. Consequently, the use of self-assessment questionnaires is a valuable method to identify "hyperreactors" [20] and a useful tool for irritancy assessment of cosmetics [21].

51.3 SENSORY TESTING METHODS

Psychophysical tests based on the report of sensation induced by topically applied chemical probes have been increasingly utilized to provide definite information on sensitive skin. These methods of sensory testing can be validated by the use of functional magnetic resonance imaging (fMRI), which represents one of the most developed forms of neuroimaging. This technique measures changes in blood flow and blood oxygenation in the brain, closely related to neural activity manifested as sensory reaction.

When nerve cells are active they consume oxygen carried by hemoglobin in red blood cells from capillaries. The local response to this oxygen utilization is an increase in blood flow to regions of increased neural activity, occurring after a delay of approximately 1–5 s. This hemodynamic response rises to a peak over 4–5 s, before falling back to baseline (and typically undershooting slightly). This leads to local changes in the relative concentration of oxyhemoglobin and deoxyhemoglobin and changes in local cerebral blood volume in addition to changes in local cerebral blood flow [22].

51.3.1 Quantitation of Cutaneous Thermal Sensation

In dermatology, thermal sensation testing analysis is the most utilized quantitative sensory testing (QST) technique [23]. It assesses function in free nerve endings and their associated small myelinated and nonmyelinated fibers. This method is able to measure quantitatively the threshold for warm and cold sensations as well as hot and cold pain.

A small device, called *thermode*, based on Peltier elements, is in contact with the subject's skin. It consists of semiconductor junctions, which produce a temperature gradient between the upper and then lower stimulator surfaces produced by an electrical current. In the center of the thermode a thermocouple records the temperature.

TSA 2001 (Medoc company, Ramat Yshai, Israel) is considered one of the most advanced portable thermal sensory testing devices.

Basically, it measures the hot or cold threshold and the suprathreshold pain magnitude (Table 51.1).

Thermal sensory test (TSA) operates between 0 and 54°C. The thermode in contact with the skin produces a stimulus whose intensity increases or decreases until the subject feels the sensation.

As the sensation is felt the subject is asked to press a button. The test is then repeated two more times to get a mean value. Using this method, artifacts can occur due to the lag time the stimulus needs to reach the brain. This inconvenience can be avoided by using relatively slow rates of increasing stimuli.

The stimulus can also be increased stepwise and the subject is asked whether or not the sensation is felt. When a positive answer is given, the stimulus is decreased by one half the initial step and so on, until no sensation is felt. The subject's response determines the intensity of the next stimulus. The limitation of this second method is that a longer performance time is required.

51.3.2 Stinging Test

Stinging test represents a method for the assessment of skin neurosensitivity. Stinging seems to be a variant of pain that develops rapidly and fades quickly any time the appropriate sensory nerve is stimulated. The test relies on the intensity

TABLE 51.1
Thermal Sensory Test

Parameters Monitored	Sensory Fibers
Warm sensation	C fiber (1–2°C above adaptation temperature)
Cold sensation	A-delta fibers (1–2°C above adaptation temperature)
Heat-induced pain	Mostly C fiber (45°C)
Cold-induced pain	Combination of both C- and A-delta fibers (10°C)

of stinging sensation induced by chemicals applied on the nasolabial fold [13]. Procedure differs depending on the chemical utilized.

51.3.2.1 Lactic Acid

After a 5–10 min facial sauna, an aqueous lactic acid solution (5 or 10% according to different methods), is rubbed with a cotton swab on the test site while an inert control substance, such as saline solution, is applied to the contralateral test site. After application, within a few minutes, a moderate to severe stinging sensation occurs for the "stingers group." Subjects are then asked to describe the intensity of the sensation using a point scale. Hyperreactors, particularly those with a positive dermatologic history, have higher scores. Using this screening procedure, 20% of the subjects exposed to 5% lactic acid in a hot, humid environment, were found to develop a stinging response [13]. Lammintausta et al. [24] confirmed these observations identifying in his study 18% of subjects as stingers. In addition, stingers were found to develop stronger reactions to materials causing nonimmunologic contact urticaria, to have increased transepidermal water loss (TEWL) and blood flow velocimetry values after application of an irritant under patch test.

51.3.2.2 Capsaicine

An alternativete test involves the application of capsaicine. Recently, a new procedure assessed by l'Oreal Recherche [25] appears to be more accurate and reliable for the diagnosis of sensitive skin. After a facial cleansing, five increasing capsaicine concentrations in 10% ethanol aqueous solution (3.16×10^{-5}%; 1×10^{-4}%; 3.16×10^{-4}%; 1×10^{-3}%; 3.16×10^{-3}%) are applied on the nasolabial folds. The application of the vehicle alone serves as control and to exclude subjects who feel any discomfort sensation prior capsaicine application. The formulation of capsaicine in hydroalcoholic solution accelerates the action of capsaicine on the face in comparison with the previously used 0.075% capsaicine emulsion, without being associated with painful sensation.

The capsaicine detection thresholds are more strongly linked to self-declared sensitive skin than the lactic acid stinging test.

51.3.2.3 Dimethylsulfoxide

The alternative application of 90% aqueous dimethylsulfoxide (DMSO) has not the same efficacy of lactic acid or capsaicine stinging test and, after application, intense burning, tender wheal, and persistent erythema often occur in stingers.

51.3.3 Nicotinate and Sodium Lauryl Sulfate Occlusion Test

A different approach to identify sensitive skin relies on vasodilation of the skin as opposed to cutaneous stinging. Methyl nicotinate, a strong vasodilator, is applied to the upper third of the ventral forearm in concentrations ranging from 1.4 to 13.7% for a 15-s period. The vasodilatory effect is assessed by observing the erythema and the use of laser Doppler velocimetry (LDV). Increased vascular reaction to methyl nicotinate was reported in subjects with sensitive skin [26]. Similar analysis can be performed following the application of various concentrations of SLS.

51.3.4 Evaluation of Itching Response

Itchy sensation seems to be mediated by a new class of C fibers with an exceptionally lower conduction velocity and insensitivity to mechanical stimuli [27].

Indeed, no explanation of the individual susceptibility to the itching sensation without any sign of coexisting dermatitis has been found. Laboratory investigations have also been limited.

An itch response can be experimentally induced by topical or intradermal injections of various substances such as proteolytic enzymes, mast cell degranulators, and vasoactive agents.

Histamine injection is one of the more common procedures: histamine dihydrochloride (100 μg in 1 mL of normal saline) is injected intradermally in one forearm. Then, after different time intervals, the subject is asked to indicate the intensity of the sensation using a predetermined scale and the duration of itch is recorded. Information is always gained by the subject's self-assessment.

A correlation between whealing and itching response produced by applying a topical 4% histamine base in a group of healthy young females, has been investigated [14]. The itching response was graded by the subjects from none to intense. The data showed that the dimensions of the wheals do not correlate with pruritus. Also, itch and sting perceptions seem to be poorly correlated.

The cumulative lactic acid sting scores were compared with the histamine itch scores in 32 young subjects; all the subjects who were stingers were also moderate to intense itchers, while 50% of the moderate itchers showed little or no stinging response [14].

Furthermore, topically applied aspirin decreases the histamine-induced itch sensation [28]. This result can be attributed to the role that prostaglandins play in pain and itch sensation [29].

Localized itching, burning, and stinging can also be a feature of nonimmunologic contact urticaria, a condition characterized by a local wheal and flare after exposure of the skin to certain agents. Nonantibody-mediated release of histamine, prostaglandins, leukotriens, substance P, and other inflammatory mediators may likely be involved in the pathogenesis of this disorder [30]. Several substances such as benzoic acid, cinnamic acid, cinnamic aldehyde, and nicotinic acid esters are capable of producing contact nonimmunologic urticaria, eliciting local edema, and erythematous reactions in half of the individuals. Provocative tests are based on an

open application of such substances and well reproduce the typical symptoms of the condition.

51.3.5 Washing and Exaggerated Immersion Tests

The aim of these tests is to identify a subpopulation with an increased tendency to produce a skin response.

In the washing test [31], subjects are asked to wash their face with a specific soap or detergent. After washing, individual sensation for tightness, burning, itching, and stinging is evaluated using a point scale previously determined.

The exaggerated immersion test is based on soaking the hands and forearms of the subjects in a solution of anionic surfactants (such as 0.35% paraffine sulfonate, 0.05% sodium laureth sulfate-2EO) at 40°C, for 20 min.

After soaking, hands and forearms are rinsed under tap water and patted dry with a paper towel. This procedure is repeated two more times, with a 2-h period between each soaking, for two consecutive days. Prior to the procedure, baseline skin parameters are evaluated. The other evaluations are taken 2 h after the third and sixth soaking and 18 h after the last soaking (recovery assessment). All of the skin parameters are performed after the subjects have rested at least 30 min at 21 ± 1°C.

51.4 BIOENGINEERING TESTS

Physiological changes indicative of sensitive skin can be detected at low levels prior to clinical disease presentation by using noninvasive bioengineering tests.

51.4.1 Transepidermal Water Loss

Transepidermal Water Loss (TEWL) is used to evaluate water loss that is not attributed to active sweating from the body through the epidermis to the environment and represent a marker of stratum corneum barrier function. TEWL assessment can be performed using different techniques (close chambers method, ventilate chambers method, and open chambers method). Measurements are based on the estimation of water pressure gradient above the skin surface. The open chamber instrument consists of a detachable measuring probe connected by a cable to a portable main signal-processing unit. The probe is provided of chambers open at both ends with relative humidity sensors (hygrosensors) paired with temperature sensors (thermistors). TEWL values ($g/m^2/h$) are calculated by the signal-processing units in the probe handle and main unit, and digitally displayed. The close chamber instrument consists of a closed cylindrical chamber containing the sensors. The humidity sensor based on a thin-film capacitative sensor is integrated to a hand-held microprocessor-controlled electronic unit provided with a digital readout for the TEWL value [32,33].

51.4.2 Corneometry

The corneometry is a method to measure stratum corneum water content (electrical measurements).

The instrument consists of a probe that should be placed to a hair-free skin surface with slight pressure. It is described as being a "capacitance" measuring device, operating at low frequency (40–75 MHz), which is sensitive to the relative dielectric constant of material in contact with the electrode surface. It estimates in about 20 ms water content of the stratum corneum to an approximate depth ranging between 60 and 100 mm, using arbitrary units.

The presence of salts or ions on the skin surface can affect the reading.

51.4.3 Laser Doppler Velocimetry

A monochromatic light from a helium-neon laser is transmitted through optical fibers to the skin. The light is reflected with Doppler-shifted frequencies from the moving blood cells in the upper dermis at the depth of ~1mm. The LDV extracts the frequency-shifted signal and derives an output proportional to the blood flow. LDV is useful in evaluating the degree of skin irritation [34].

51.4.4 Colorimetry

Surface color may be quantified using the Commission Internationale de L'Eclairage (CIE) system of tristimulus values. The device utilizes silicon photocells. The measuring head of these units contains a high-power pulsed xenon arc lamp, which provides two CIE illuminant standards. The color is expressed in a three-dimensional space. The coordinates are expressed as L* (brightness), a* value (color range from green to red), and b* value (color range from blue to yellow). The a* value, related to skin erythema, increases in relation to irritation and skin damage.

51.4.5 Corneosurfametry

This method [35] investigates the interaction of surfactants with the human stratum corneum. It is performed as follows: cyanoacrilate skin surface stripping (CSSS) is taken from the volar aspect of the forearm and sprayed with the surfactant to be tested. After 2 hs the sample is rinsed with tap water and stained with basic fuchsin and toluidine blue dyes for 3 min. After rinsing and drying, the sample is placed on a white reference plate and measured by reflectance colorimetry (Chroma Meter@ CR200, Minolta, Osaka, Japan).

The index of redness (CIM = Luminacy L* – Chroma C*) is taken as a parameter of the irritation caused by the surfactant. This index has a value of 68 ± 4 when water alone is sprayed on the sample and decreases when surfactant is tested, with stronger surfactants lowering the values.

Piérard et al. [36], testing different shampoo formulations in volunteers with sensitive skin, demonstrated that corneosurfametry correlates well with *in vivo* testing. A significant negative correlation ($p < .001$) was found between values of colorimetric index of mildness (CIM) and the skin compatibility parameters (SCP) that include a global evaluation of the colorimetric erythemal index (CEI), and the TEWL differential, both expressed in the same order of magnitude.

In the same study corneosurfametry showed less inter-individual variability than *in vivo* testing, allowing a better discrimination among mild products.

An interesting finding showed that sensitive skin is not a single condition. Goffin [37] hypothesized that the response of the stratum corneum to an environmental threat might be impaired in different groups of subjects experiencing sensitive skin. Data of the corneosurfametry performed after testing eight different house-cleaning products, showed that the overall stratum corneum reactivity, as calculated by the average values of the corneosurfametry index (CSMI) and the CIM, is significantly different ($p < .01$) between detergent-sensitive skin and both nonsensitive and climate/fabric-sensitive skin, as well.

51.4.6 Irregularity Skin Index

Irregularity skin index (ISI) can contribute to the identification of subjects with sensitive skin.

In a recent study [38] conducted on 243 subjects positive to the lactic acid stinging test, slides of cyanoacrylate skin surface stripping (CSSS), obtained from the volar aspect of the forearm, were examined by means of a computer-assisted fast Fourier transform (FFT) to determine the skin surface microrelief. Acquisition of the images was performed by a stereomicroscope connected to an analogical video camera. The results confirmed a significant correlation ($p < .001$) between intensity of symptoms in "stingers" and ISI. This procedure represents a valuable and promising tool for the study and the diagnosis of sensitive skin.

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52 Test Methods for Allergic Contact Dermatitis in Animals

Georg Klecak

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52.1 INTRODUCTION

Among the dermatotoxicologic community working on immune skin effects manifested as *allergic contact dermatitis*, the proven effective strategy has been to develop and apply animal assays for identification of chemical xenobiotics with allergenic potential and to assess the sensitization hazard potential of these environmental contactants for human beings. Since 1935 (Landsteiner and Jacobs, 1935), the guinea pig has represented the reference animal model.

Besides the official regulatory guinea pig methods listed in Appendix 3 of the OECD test guidelines (No. 406; OECD, 1992), another category of "nonregulatory"/investigative testing strategies is represented by various modifications of the Draize test, the guinea pig maximization test, and the split adjuvant test (Figure 52.1).

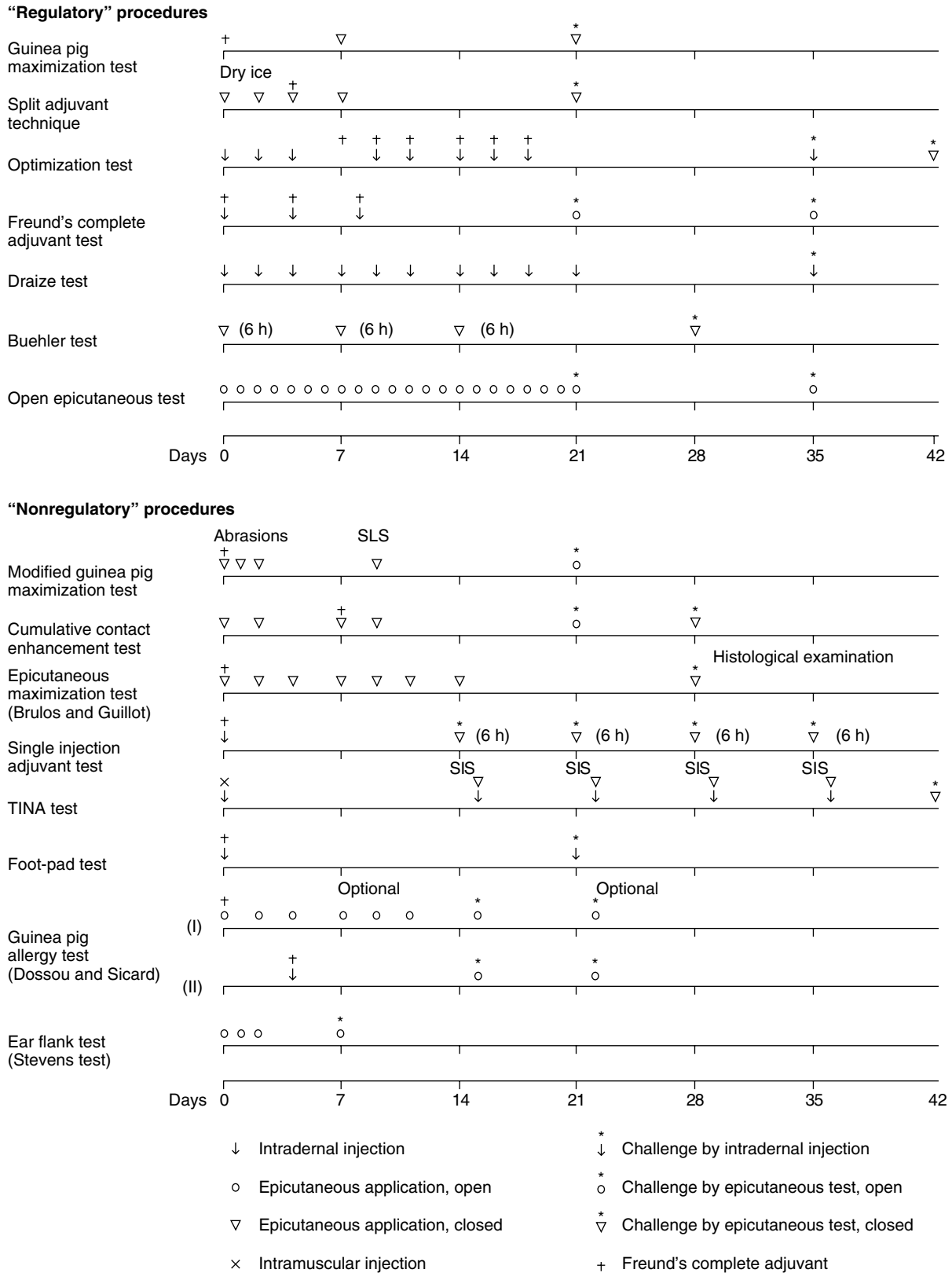


FIGURE 52.1 Guinea pig contact allergy tests: procedures for induction and challenge.

The main purpose of this chapter is to convey to the reader a general understanding of the rationale, design, and predictive value of each particular testing strategy, reviewed here.

The common principle of all these methods is to initiate exposure(s) of a test article or its test samples to the same skin site or area (*induction phase*), which after a rest period of at least 7 days is followed by a *challenge exposure* of the article or of its test sample(s) to a virgin skin site or area.

Animal bioassay data are currently generated from experimental designs, outlined in various testing guidelines issued by OECD (1981, 1992), EEC (1983), U.S. Environmental Protection Agency/TSCA (1985), U.S. Environmental Protection Agency/FIFRA (1984), OSHA (1987), CTFA (1981), and Japan/MAFF (1985) for allergenicity assessment of single chemical and "end-use" products. Generally, guinea pig assays vary with respect to administration route and mode, exposed skin surface conditions, frequency, number and duration of exposures, and dosing of a test article (Table 52.1). Variations among testing protocols give rise to differences in their sensitivity and predictivity (Marzulli and Maguire, 1982). The key factors influencing the outcome of a skin sensitization assay in guinea pigs are of biological (genetic), chemical, and operative origin.

The main biological factor is the choice of a suitable guinea pig strain. Guinea pigs of Hartley, Pirbright, or Himalayan white strains, weighing 350–400 g, are known to be "good responders" in spite of differences in response to some human contact allergens (Chase, 1941; Polak et al., 1968; Parker et al., 1975; Rockwell, 1955; Stampf and Benezra, 1982). Only with exposure to moderate or weak contact allergens do the operative factors have an effect on the sensitization rate of test animals (Goodfrey and Baer, 1991). Of these, the following should be considered:

1. Mode and manner of exposure—Repeated, intradermal, or occlusive applications onto the same skin site or area are more efficient for induction of contact allergy than the open application (Kligman, 1966b).
2. Skin conditions—Application over slightly inflamed, scarified, or sodium lauryl sulfate (SLS) pretreated skin site improves the sensitizing rate of weak sensitizers (Vinson et al., 1965; Baker, 1968; Kligman, 1966a).
3. Use of "adjuvants"—Employment of Freund's complete adjuvant (FCA) for induction has maximal stimulating effect on the immune mechanism and may convert chemicals with poor allergenic properties into moderate or strong sensitizers (Goodfrey and Baer, 1971; Andersen, 1985).
4. Dosing—The use of higher concentrations and higher dosing increases the sensitization rate (Magnusson and Kligman, 1970; Marzulli and Maibach, 1974; Christensen et al., 1984).
5. Vehicle choice—Marked differences in the intensity and frequency of skin reactions were found when different vehicles were used (van der Walle et al., 1982; Björkner and Niklasson, 1984).
6. Occlusion—An occlusion time of 24 h or longer results in uniform immune responses reduces the vehicle effect, and yields a lower response level than that found by open exposure (Magnusson and Hersle, 1965; Pirilä, 1974).
7. Area of sensitizing exposure—The size of skin surface patches used for induction should be >4 cm^2, while the appropriate size for challenge patches would be 2–4 cm^2 (White et al., 1986).
8. Dose–response effect—Graded skin response is induced and elicited by using graded concentrations in sensitization protocol, enabling determination of end points as minimal sensitizing eliciting and maximal nonsensitizing/noneliciting concentration level (Bronaugh et al., 1994).
9. Animal—Care and housing conditions (Andersen, 1993; U.S. Department of Health, Education, and Welfare, 1978).

52.2 GENERAL PRINCIPLES

The following points belong to the obligatory (elementary) parts of every study protocol for allergenicity assessment involving experimental animals.

52.2.1 Preliminary Irritation Test

A special group of four to six animals is used to establish a suitable (moderate irritant) concentration for intradermal or epicutaneous induction treatment and the highest nonirritant one for challenging. In addition, care has to be taken that FCA treatment may lower the threshold for the primary nonirritant concentration in test animals (Magnusson, 1980).

52.2.2 Controls

It is necessary to include negative or vehicle control animals in the test strategy to ensure that the challenge reactions, seen in test animals, are of allergic origin and not due to skin irritancy. Controls have to be treated in exactly the same manner as the test animals, except that during the induction phase the use of a test article is omitted.

For rechallenge or cross-test, it is essential to incorporate a new set of four to six naive control animals. The use of positive controls is required to validate the test procedure and system (guinea pig colony) twice a year using one of the moderate and weak contact sensitizers (hydroxcitronellal, neomycine sulfate, benzocaine).

52.2.3 Evaluation

Evaluation of skin reactions is usually performed by visual scoring of erythema, edema, and other clinical changes of

skin using various grading scales. Magnusson and Kligman (1969, 1970) prefer to use an ordinal scale:

−			=	no visible change
+			=	patchy erythema
+	+		=	confluent erythema
+	+	+	=	confluent erythema and edema

It is possible to assign numerical values to these gradings. An exceptional case is the optimization test, in which "reaction volume" in micro liters serves as the parameter for evaluation of skin reactions.

The scoring system is of relative value, but comparison of frequency, intensity, and duration of reactions elicited on test and control animals generally permits an unequivocal decision as to whether sensitization has occurred or not.

52.2.4 Classification

A potential contact sensitizer is classified as any article that produces in a nonadjuvant assay at least 15% of test animals with allergic contact dermatitis. A negative result is one in which no skin reaction was elicited in any of the experimental animals. All other results would be classified as questionable and indicate the need for rechallenging or study repetition.

Based on the results of an adjuvant test, each test article that sensitizes at least 8% of test animals is classified as a sensitizer. In case of a lower percentage of sensitized animals, a rechallenge is recommended for confirmation of challenge results (Figure 52.1).

52.3 THE GUINEA PIG MAXIMIZATION TEST

In the guinea pig maximization test (GPMT), as described by Magnusson and Kligman (1969, 1970), 20 test and 10–20 control guinea pigs are used (Figure 52.2).

The induction, consisting of two phases, is initiated (day 0) by paired intradermal injections (0.1 mL each) into the clipped and shaved shoulder region of test animals of

1. Complete Freund's adjuvant (FCA)
2. Test article in suitable vehicle (water, paraffin oil, propylene glycol)
3. Mixture of dissolved or suspended test article with FCA (1:1)

On day 7, for boosting, a topical occlusive patch is applied for 48 h on the shoulder region, which has been clipped 24 h before.

For induction of sensitization, the use of a mildly or moderately irritating test concentration is recommended. When nonirritating test articles are involved, pretreatment of the freshly clipped shoulder region with 10% SLS is indicated on day 6.

Challenge is performed on day 21. On the left flank of all animals a skin site of 4 cm^2 is shaved, to which the test article is applied in suitable vehicle at primary nonirritating concentration(s) using a 24-h occlusive "patch unit" with Finn chambers, for example. The vehicle may be simultaneously tested, if indicated. The challenge reactions are examined 24 and 48 h after removal of the patch, scored according to a standard rating scale, and the classification of allergenic potential is graded from none to extreme. Rechallenge or cross-test may follow at weekly intervals, always on contralateral flanks.

Control animals are treated similar to test animals, except that during the induction phase the test article is omitted.

The GPMT is a very sensitive procedure for allergenicity screening of test articles (Maurer et al., 1978; Kero and Hannuksela, 1980; Stampf and Benezra, 1982; Andersen, 1985, 1993; Andersen et al., 1984, 1985; Andersen and Maibach, 1985a,b; Wahlberg and Boman, 1985) with a tendency to overestimate the potency of many weak, mild, and moderate human sensitizers. Even if its experimental data are less suitable for sensitization hazard calculation related to intended, accidental, or occasional exposure of human skin to various environmental allergens, the GPMT is strongly recommended as a legislative method.

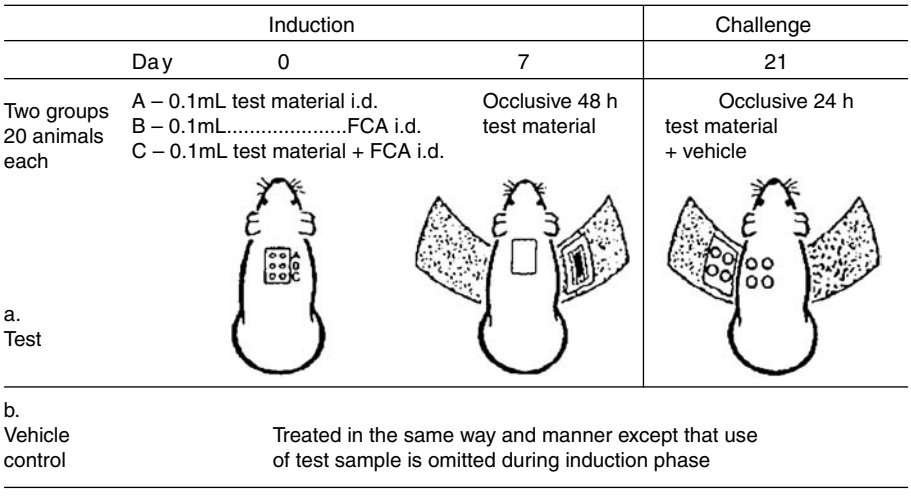


FIGURE 52.2 Guinea pig maximization test (GPMT).

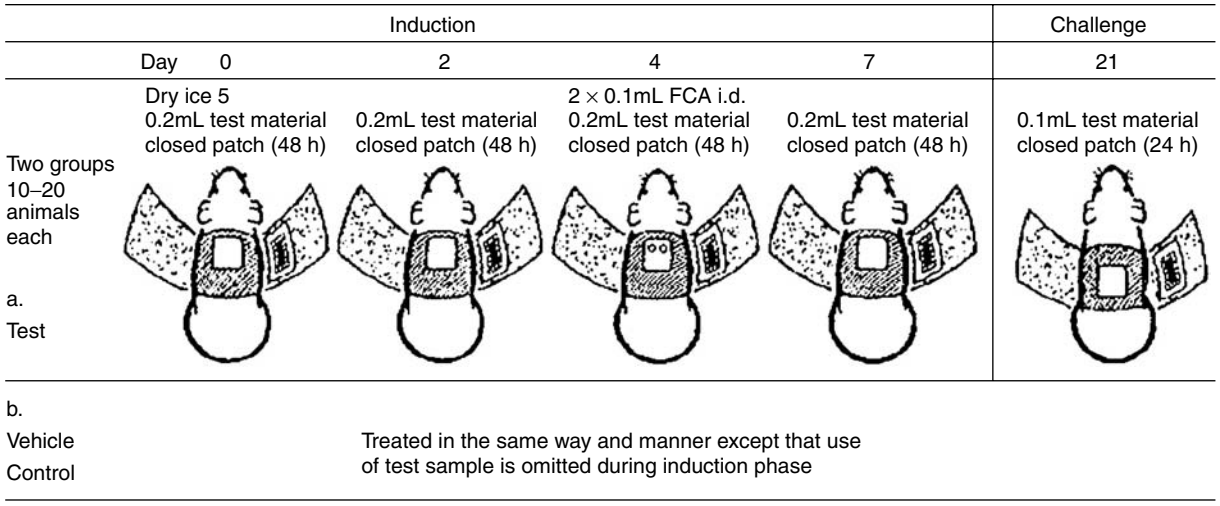


FIGURE 52.3 Split-adjuvant technique.

Due to limits and deficiencies of this testing strategy, various modifications of the GPMT are utilized to improve its predictivity (Rochas et al., 1977; Kozuka et al., 1981; Sato et al., 1981; Guillot and Gonnet, 1985; Maurer and Hess, 1989). The use of fewer test animals was recommended by Hofmann et al. (1987) and Shillaker et al. (1989).

52.4 SPLIT-ADJUVANT TECHNIQUE

In this assay (Figure 52.3), the test article and FCA are administered separately (Maguire, 1973, 1975, 1985; Maguire and Cipriano, 1985), and two groups of 10–20 guinea pigs each are involved.

52.4.1 Induction

On day 0, the skin of the suprascapular region is shaved to remove hair and loose keratin. Thereafter, a window dressing is fixed over this skin site. The induction site of 2 cm^2 is exposed to "dry ice" for a minimum of 5 s prior to the application of 0.2 mL semisolid or 0.1 mL liquid test sample, covered with filter paper, fixed with adhesive tape, and kept under occlusion for 48 h. This procedure is repeated every other day up to a total of four induction treatments on day 4, prior to topical application of the test sample, two intradermal injections of 0.1 mL FCA are administered symmetrical into the induction site. On day 9 the dressing is removed.

52.4.2 Challenge

On day 21, challenge is performed by 24 h-occlusive or open patch test application of 0.5 mL semisolid test article, or 0.1 mL if liquids are tested, to a virgin clipped skin site on the dorsal back measuring 2 cm^2. Controls are treated similarly, except that test article administration is omitted during the induction phase. Reading and scoring of elicited skin reactions are done on days 22, 23, and 24. Rechallenge or cross-tests can follow at intervals of 10 days.

This test protocol is designed for the testing of chemicals as well as "end use" products. It is somewhat less sensitive than the GPMT. Its performance is rather complicated, and is stressful for animals due to the use of window dressing during the induction phase.

In the modified Maguire Test (MM) (Figure 52.4) by Prince and Prince (1977) or Rao et al. (1981) the use of occluded patches is replaced by open application of 0.2 mL of the test sample onto the reexcoriated (with sandpaper or tape) skin site in the suprascapular region. The MM is carried out under test conditions that are closer to the "in-use" situation, due to separate and open application of allergens. The database is relatively new.

52.5 THE OPTIMIZATION TEST

As described by Maurer (1974, 1985) and Maurer et al. (1978, 1980), the optimization test (OPT) protocol involves two groups of 20 test and control animals (Figure 52.5).

52.5.1 Sequence of Induction

On day 0, two intradermal injections of the test article, dissolved or suspended (0.1%) in suitable vehicle, are administered into a shaved flank (0.05 mL) and dorsal area (0.1 mL) of the test animals.

On days 2 and 4, injections (0.1 mL) of a 0.1% test sample are given into the back area. Approximately 24 h after each of the four initial intradermal injections during the first week of induction, the reaction sites are depilated and the skin reactions are examined by measuring and multiplying the two largest perpendicular diameters and the skin-fold thickness to obtain the "reaction volume" and calculate skin irritation threshold for each animal.

On day 6, an intracutaneous dose (0.1 mL) of a 1:1 emulsion or mixture of a dissolved or suspended test article in vehicle with FCA is administered into the clipped nuchal

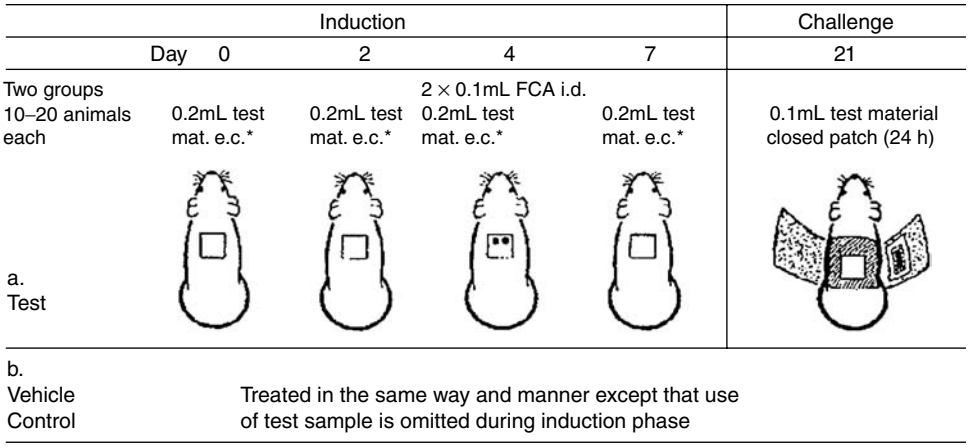


FIGURE 52.4 Modified split-adjuvant test.

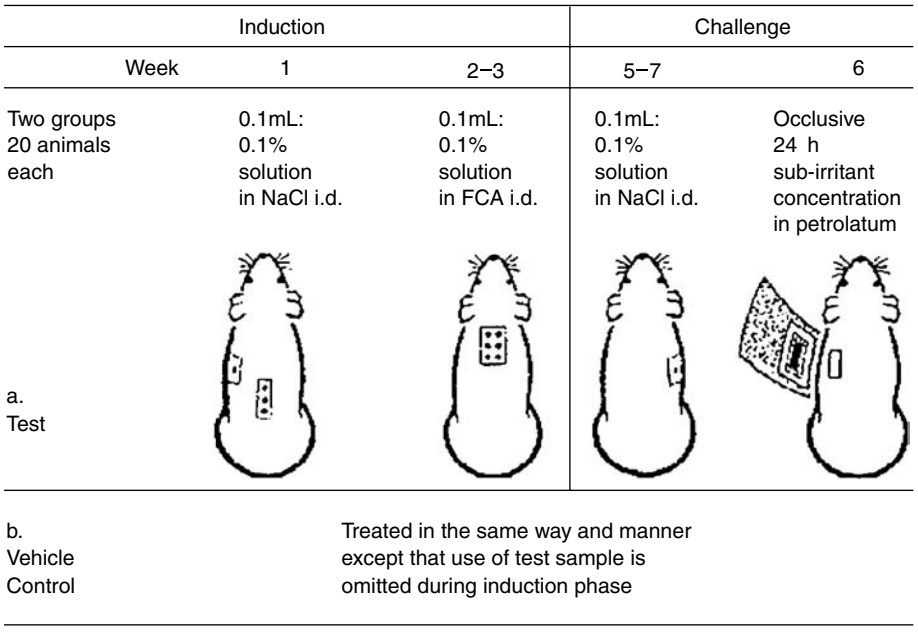


FIGURE 52.5 Optimization test.

region. This intracutaneous administration is repeated on days 8, 10, 12, 14, and 16.

52.5.2 First Challenge

About 2 weeks after the last induction, the 0.1% test sample is injected with 0.05 mL into a virgin skin area on the contralateral flank, and the 24-h skin reactions are measured.

52.5.3 Assessment

If the reaction volume of the challenge injection is greater than the skin irritation threshold, the animal is termed as sensitized (positive). The second parameter represents skin reactions, which were induced and elicited in control animals. For final evaluation the number of "positive" test animals is compared with the number of control animals that show nonspecific reactions of comparable intensity, using the exact Fisher test.

A second challenge can be performed 1 week later if the results of the first challenge are negative or marginal, using a maximum nonirritant concentration of the test article in suitable vehicle for open or occlusive exposure. Skin reactions are examined 24, 48, or 72 h after challenge and classified according to a standard rating scale.

Control animals are treated in the same manner as test animals, except that during induction the administration of test article is omitted.

Variation in reactions to epidermal challenge is taken into account by means of statistical group comparison and the Fisher test.

The optimization test and the maximization method are almost equally efficient (Maurer et al., 1980; Stampf

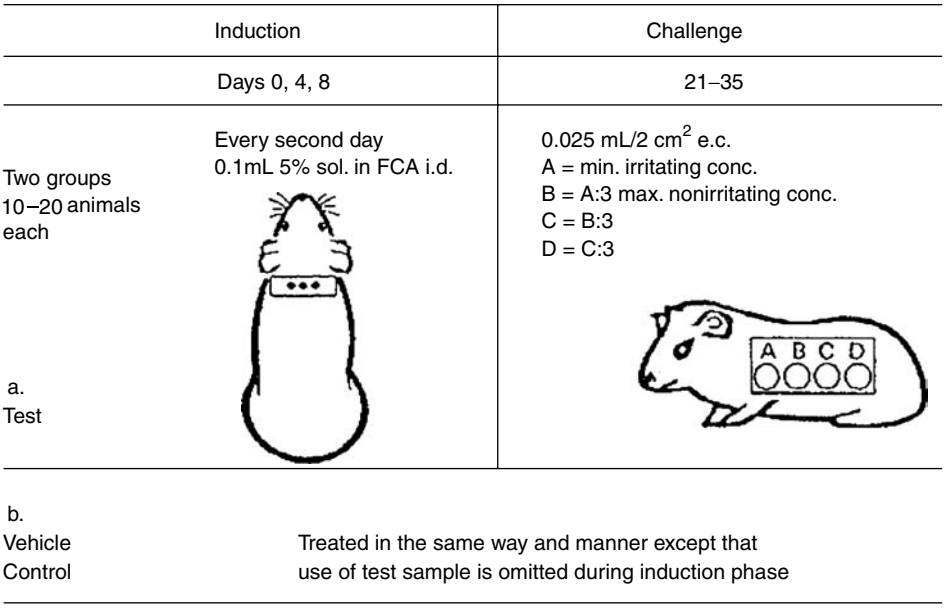


FIGURE 52.6 FCA test.

et al., 1982; Stampf and Benezra, 1982). Both test protocols may overclassify some chemicals in terms of their sensitizing potential for humans and share similar merits and deficiencies.

The OPT has a database of industrial chemicals, warranting more flexible objective assessment of skin responses.

52.6 FREUND'S COMPLETE ADJUVANT TEST

Freund's complete adjuvant test (FCAT; Figure 52.6) uses a test and a control group of 10–20 guinea pigs each (Klecak et al., 1977; Klecak, 1982, 1985).

Induction is performed in the suprascapular region. A skin site of approximately 6×2 cm is shaved and an intradermal injection of 0.1 mL of a 1:1 mixture or emulsion of FCA and the dissolved test article at a concentration of 5% or less is administered on days 0, 4, and 8.

Challenge is performed on day 21 on clipped flank skin using open application. Up to six concentrations (the minimal irritating one and its 1:3 nonirritating dilutions) of the test article in suitable vehicle may be used and are applied to a test site of 2 cm^2 at a dose of 0.025 mL (liquids) or 0.01 mL (semisolids), which had been marked with a circular stamp.

Control animals are treated similarly, except that the use of the test article is omitted during induction phase.

Challenge reactions are examined 24, 48, and 72 h after challenging and graded according to a standard rating scale. Rechallenge and cross test may be performed in intervals of 10–14 days on the contralateral flank. For each rechallenge or cross-test at least four new control animals need to be used.

The FCAT is easy to perform, economical, and as sensitive as the GPMT. It is not suitable for testing insoluble test articles or end-use products, and tends to overestimate the allergenic potential of weak and moderate human contact allergens. Nevertheless, it yields quantitative data in the form of a threshold concentration for elicitation of allergic contact dermatitis, based on the endpoint determination (Stampf et al., 1982; van der Walle and Bensink, 1982; van der Walle et al., 1982; Klecak, 1985; Avalos et al., 1989; Gäfvert, 1994).

The FCA test in sequential combination with the open epicutaneous test represents an adequate program for detection of strong and moderate, as well as weak, allergenic potential of single chemicals and injectible end-use products. Both animal assays (FCAT and OET) yield data of predictive value regarding actual risk estimate of contact sensitization in humans under anticipated use conditions and occasional or accidental exposure.

52.7 THE MODIFIED DRAIZE TEST

The aim of all modifications of the Draize test (Draize, 1955, 1959) is to enhance the sensitivity of this animal assay for detecting weaker skin sensitizers by increasing the test concentration as high as to cause moderate irritant skin responses at maximum (Voss, 1958), including control animals for challenging (Maurer et al., 1978), replacement of intradermal administration by open application (Prince and Prince, 1977), increased frequency of exposures by rechallenge, or repetition of the whole study course in the same test animal group (double Draize test), or shortening the duration of the induction to 1-day treatment by administration of four intradermal injections at sites overlying the axillary and inguinal lymph nodes (Sharp, 1978).

The procedure of the modified Draize test (MDT), according to Johnson and Goodwin (1985), consists of two parts, and involves two groups of 10 guinea pigs each (Figure 52.7).

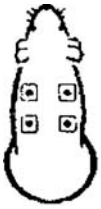
Induction		Challenge
Day 0		14
Two groups 10 animals each	4 × 0.1mL sol. i.d 2.5 times ICC	0.1mL sol. e.c. + 0.1mL sol. i.d.
a. Test		
b. Vehicle Control	Treated in the same way and manner except that use of test sample is omitted during induction phase	

FIGURE 52.7 Modified Draize test.

52.7.1 Part I

52.7.1.1 Induction Phase

Ten test animals are used. On day 0, four intradermal administrations of the test article at a dose of 0.1 mL and at a concentration corresponding to 2.5 times the intradermal challenge concentration (ICC, may cause slight but perceptible irritation on guinea pig skin) on the clipped skin site overlying both axillary and inguinal lymph nodes are employed.

Resulting 24-h skin reactions are examined, their intensity graded (erythema and edema), and the average reaction size evaluated based on the measurement of the longitudinal and lateral axes diameters of each of the four skin reactions.

52.7.1.2 Challenge Phase

On day 14, each of the experimental animals is challenged using intradermal administration of 0.1 mL of test articles in suitable solvents at a nonirritant or slightly irritant concentration at maximum on one clipped flank, and open epicutaneously on the opposite clipped flank with 0.1 mL of the test samples at primary nonirritant concentration to a circular test site of about 8 cm^2. The 24-h reactions are examined, graded, and their size evaluated. Confirmation rechallenge may follow on days 21 and 28. For each rechallenge 10 control animals, which had been treated with FCA for induction solely, are challenged similar to the test animals.

52.7.2 Part II

If both challenge tests of Part I are negative (no evidence that skin sensitization occurred in test animals), a second set of intradermal injections is administered on day 35. The challenge procedure is similar to the one described for Part I, but confirmation rechallenge is done intradermally and epicutaneously in weekly intervals. New control animals have to be involved.

This technique is suitable for testing of soluble or suspensible chemicals exclusively, and is significantly more sensitive than the Draize guinea pig test, though still less sensitive than the GPMT and the FCAT.

52.8 THE BUEHLER TEST

The standardized protocol (Figure 52.8) involves a group of 20 test and one or more groups of 10 control animals each (Bühler, 1965, 1982, 1985; Bühler and Griffith, 1975; Griffith and Bühler, 1969; Ritz and Bühler, 1980).

The induction phase is performed by 1 or 3 weekly occlusive application(s) of the test sample at a slight to moderately irritating concentration for a minimum of 3 or a maximum of 9 weeks. The induction skin site of approximately 8 cm^2 on the animal's left shoulder is clipped 24 h before the guinea pigs are placed into restrainers and, using 4-cm^2 occlusive patches for 6 h, Webril pads with 0.4 mL test sample or Hill-Top chambers with 0.2 mL test sample at the highest possible (moderate irritating) or anticipated use concentration, are fixed to the skin.

For the challenge phase, when a rest period of 14 days has passed after the last induction exposure, test animals and 10 control animals are challenged by the application of 0.2–0.5 mL of the test sample at primary nonirritating concentration(s) to a naive clipped back skin site under an occlusive patch for 6 h.

During the induction and challenge procedures, the animals are kept in a specially designed restrainer, which prevents their movement and enables attachment of the occlusive patch with a rubber dental dam, slightly pulled and fastened to the restrainer.

The observations and gradings of elicited skin reactions on previously depilated test sites are done 24 and 48 h after challenge.

Single or multiple rechallenge(s) or cross-test(s) may follow at intervals of 1 or 2 weeks, always to a virgin skin site on the animal's back. If indicated, a vehicle may be used additionally.

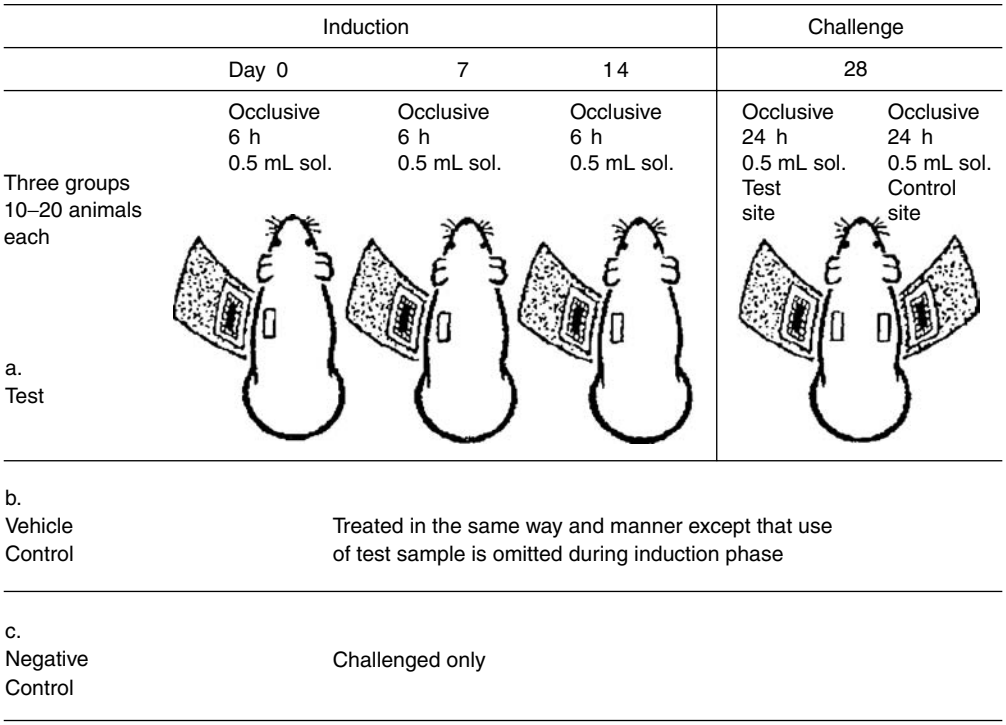


FIGURE 52.8 Buehler test.

Control animals are treated in the same manner, except that the use of the test article for induction is omitted.

For evaluation, two parameters are used: The *incidence index* is an expression of the number of responding animals out of the total test animals, while the severity index is calculated from the total sum of 24-h and 48-h reaction grades divided by the number of animals exposed.

The Buehler test is suitable for testing single chemical as well as end-use products. This assay is uneconomical, time-consuming, and the validity of results is usually limited to use concentration.

Nevertheless, the Buehler test is often recommended as a legislative method, especially in the United States.

52.9 THE OPEN EPICUTANEOUS TEST

In the open epicutaneous test (OET), a test article and its sample(s) are applied epicutaneously uncovered to the test site with intact skin surface (Figure 52.9). Constant volumes per square centimeter of each test sample are applied to standard areas of the clipped flank skin during the induction and elicitation phase of the assay. For OET, at least six guinea pigs are utilized for every test concentration group, while end-use products are tested in 20 animals. Ten guinea pigs constitute a control group (Klecak et al., 1977).

Induction requires daily applications on 5 or 7 consecutive days per week of 0.1 mL of the neat test article or its progressively diluted (3:1) solutions, emulsions, or suspensions, usually to the same skin area of 8 cm^2 on a clipped flank, with 20 exposures in total. In cases where moderate to strong skin reactions are induced on test animals, the application sites are changed.

For challenge, on day 21 or 29 all experimental animals are treated on the contra-lateral clipped flank with the test article or its dilutions at the minimal irritating and some lower primary nonirritant concentrations at a dose of 0.025 mL (solutions) or 0.01 mL (semisolids) per circular test site of about 2 cm^2 in size. Vehicle may be involved at challenge, if indicated.

Control animals are treated similarly, except that the use of test article is omitted during induction.

The reactions are read and recorded 24, 48, and 72 h after challenging. Rechallenge(s) or cross-tests can follow at intervals of 10–14 days, always on the contralateral flank.

This technique shows no limitations in terms of physicochemical properties of test articles, has high flexibility, and enables prediction of health-risk calculation related to intended, occasional, or accidental exposure of human skin to various types of potential contact allergens, since dose-related experimental data are obtained (Klecak, 1982).

The OET fails when borderline sensitizers are tested or test articles with limited skin penetration capability are involved. A complementary testing program, combining either the FCAT or GPMT with the OET, is adequate to define conditions under which a contact allergen can act as a skin sensitizer in humans or not, a crucial point in predictive testing (Stampf et al., 1982; van der Walle et al., 1982; van der Walle and Bensink, 1982; Kero and Hannuksela, 1980). A good correlation exists between HRIPT and OET results (Klecak, 1985).

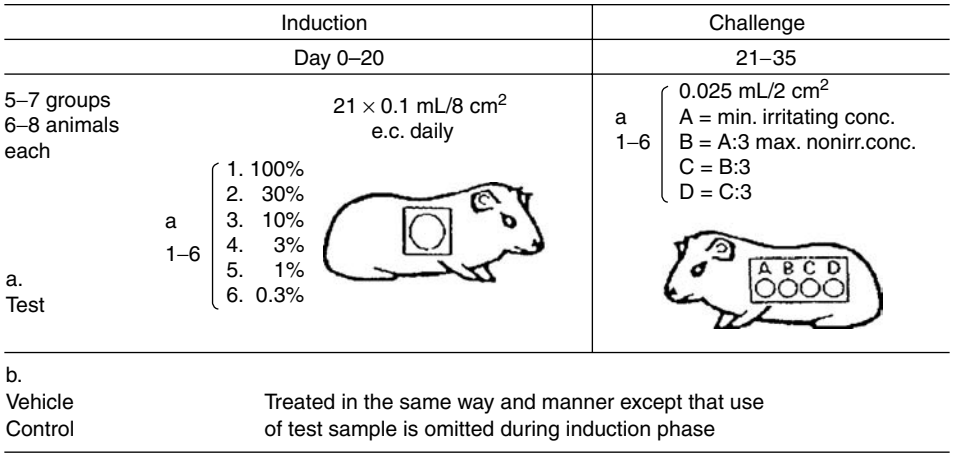


FIGURE 52.9 Open epicutaneous test.

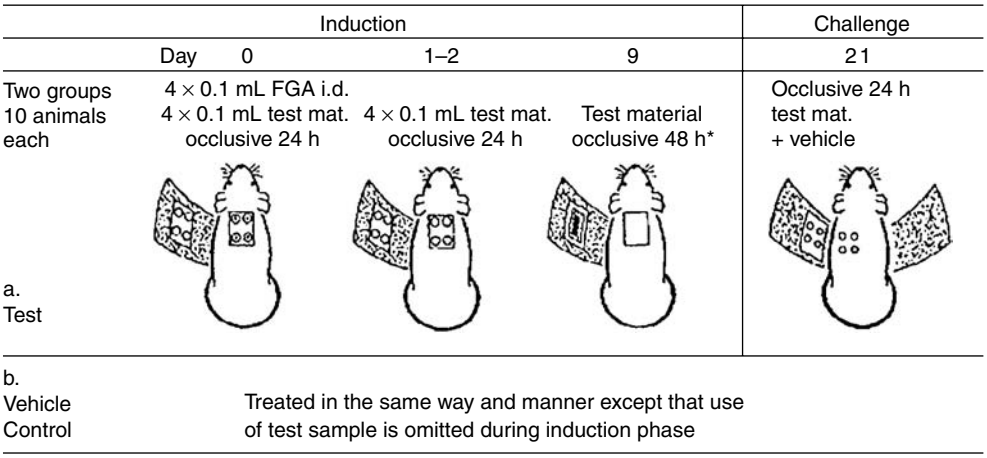


FIGURE 52.10 Modified guinea pig maximization test.

52.10 MODIFIED GUINEA PIG MAXIMIZATION TEST

There are several drawbacks to the modified guinea pig maximization text (GPMT). For example, intradermal administration is an unnatural exposure route for a contact allergen, often resulting in overestimation of allergenicity of test articles. It is not suitable for testing poorly soluble or insoluble test articles and end-use products, since these are not injectible.

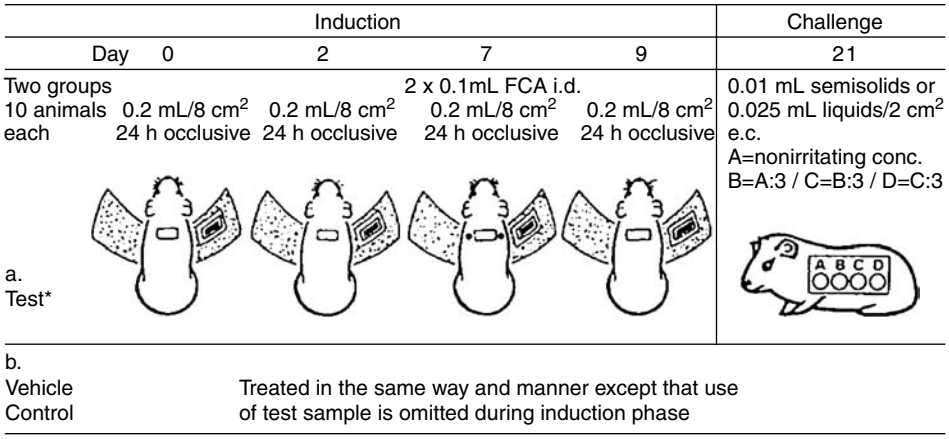
The modified protocol (Sato et al., 1981, 1984; Sato, 1985) differs from the GPMT shown in Figure 52.10 in that for the first induction phase on 10 test animals, four injections of 0.1 mL FCA emulsified with bidistilled water (1:1) are administered intradermally into the comers of a 4 × 2 cm skin site on the clipped nuchal skin area. Thereafter, the sites of injection are abraded by superficial incisions and each of them is covered with a circular lint patch of 1.5 cm in diameter with 0.1 mL (0.1 g) of the test sample, occlusively fixed in place for 24 h. Skin abrasion and application of the four patches under occlusion are repeated on the next 2 days.

Approximately 24 h prior to the second induction treatment by a 48-h occlusive patch test of 2.5 × 4 cm with the test sample on day 9, the nuchal area of animals is clipped, shaved, and the skin treated with 10% SLS in petrolatum if indicated.

Another variant of the first induction phase, as proposed by Maurer and Hess (1989), consists of four intradermal injections of a 1:1 FCA/saline emulsion, followed by a 24-h occlusive application of the test article in white petrolatum or of a neat end-use product.

The challenge is performed on day 21, using occlusive patch or open (uncovered) application, on the clipped flank skin. For the 24-h occlusive patch, the test sample is incorporated into petrolatum or other suitable vehicle and applied at a dose of 50 mg to the lint patch of 2.5 × 4.0 cm, or a Finn chamber "patch unit" can be involved, which is occlusively fixed to the test site. For the open challenge, 0.01 mL (10 mg) of the test sample is applied directly to a circular test site 1.5 cm in diameter.

The challenge sites are evaluated and scored 24 and 48 h after initiation of the challenge exposure. If appropriate, rechallenge and cross-tests are done in weekly intervals on



* Groups treated with different concentrations, e.g., 100%, 30%, 10%, 3%, and so on.

FIGURE 52.11 Cumulative contact enhancement test.

contralateral flanks. The 10 control animals are treated in the same way as the test animals, except that during induction, application of the test article is omitted. This testing strategy is suitable for allergenicity assessment of neat chemicals and end-use products. It is sensitive enough to identify weak contact allergens, and yields quantitative data in the form of threshold concentrations, which improves its predictive value.

52.11 THE CUMULATIVE CONTACT ENHANCEMENT TEST

The test protocol for this animal bioassay (Figure 52.11) involves separate application of Freund's complete adjuvant and the test articles (Tsuchiya et al., 1982). The epidermal application of the test sample for induction simulates more accurately common exposure conditions to a potential environmental contact allergen. Two groups of 10 guinea pigs are used at minimum.

For induction, the test animals are exposed to the test sample on days 0, 2, 7, and 9, at a dose of 0.2 mL of liquids or 0.1 mL of semisolids, applied to a linen patch of 2×4 cm, and fixed under a 24-h occlusive patch to the clipped suprascapular area. On day 7, prior to the patch application, 2×0.1 mL of Freund's complete adjuvant is administered intradermally parallel on each side of the application area.

For the challenge test, on day 21 one animal flank is shaved and 0.01 mL (semi-solids) or 0.025 mL (liquids) of the test sample, at nonirritating concentrations, is applied to circular skin area(s) of 2 cm in diameter using open application or 24-h occlusive patches. Rechallenge(s) may be performed to the contralateral flank in 7–10 days of sequence. The 24-, 48-, and 72-h skin reactions are scored according to a standard rating scale. Controls are treated in the same manner, except that the use of test article is omitted during the induction phase.

The cumulative contact enhancement test (CCET) is specified as a suitable tool to categorize contact allergens as weak, moderate, or severe skin sensitizers (Tsuchiya et al., 1985; Scheper et al., 1990; Gäfvert, 1994).

This test strategy is recommended to screen allergenicity of poorly soluble or insoluble chemicals as well as end-use products. The database is relatively new.

Two short-period or abbreviated variants of the CCET were reported by Kashima et al. (1993a,b), which are designated as the AP 2 test (FCA intradermally and 24-h occlusive patch on day 0 and 4 for induction), and the CAP 2 test (cyclophosphamide i.p. on day 0 and FCA intradermally with occlusive patch on days 3 and 7). In both variants, the challenge test is performed on day 14 followed by scoring of elicited skin reactions as described by the CCET.

Both methods shorten the test, and in the CAP 2 test the cyclophosphamide effectively enhances the skin reaction of the guinea pig to weak allergens.

52.12 THE EPICUTANEOUS MAXIMIZATION TEST

This test protocol is designed to assess the allergenic potential of end-use products and their single compounds (Brulos et al., 1977). It includes epicutaneous application of a test article, using occlusive patch and intradermal administration of complete Freund's adjuvant. Macroscopic readings of doubtful challenge skin reactions are evaluated microscopically; 20 guinea pigs are involved (Figure 52.12).

The preliminary test, initiated prior to the main study, is performed as follows: On day 0, the test article is applied as such or diluted at a dose of 0.5 mL (liquids, semisolids) or 0.5 g (moistened solids), using the 48-h occlusive patch on a shaved skin area of 2 cm^2 behind the left shoulder blade. Readings of the treated skin sites are performed 1, 6, 24, and 48 h after removal of the patch, and skin reactions are scored. Animals showing moderate to severe skin responses (strong reactors) must be replaced.

For the main study, details concerning the induction as well as the challenge phase of this experimental design are best reproduced in Figure 52.12, as completed by Guillot and Gonnet (1985).

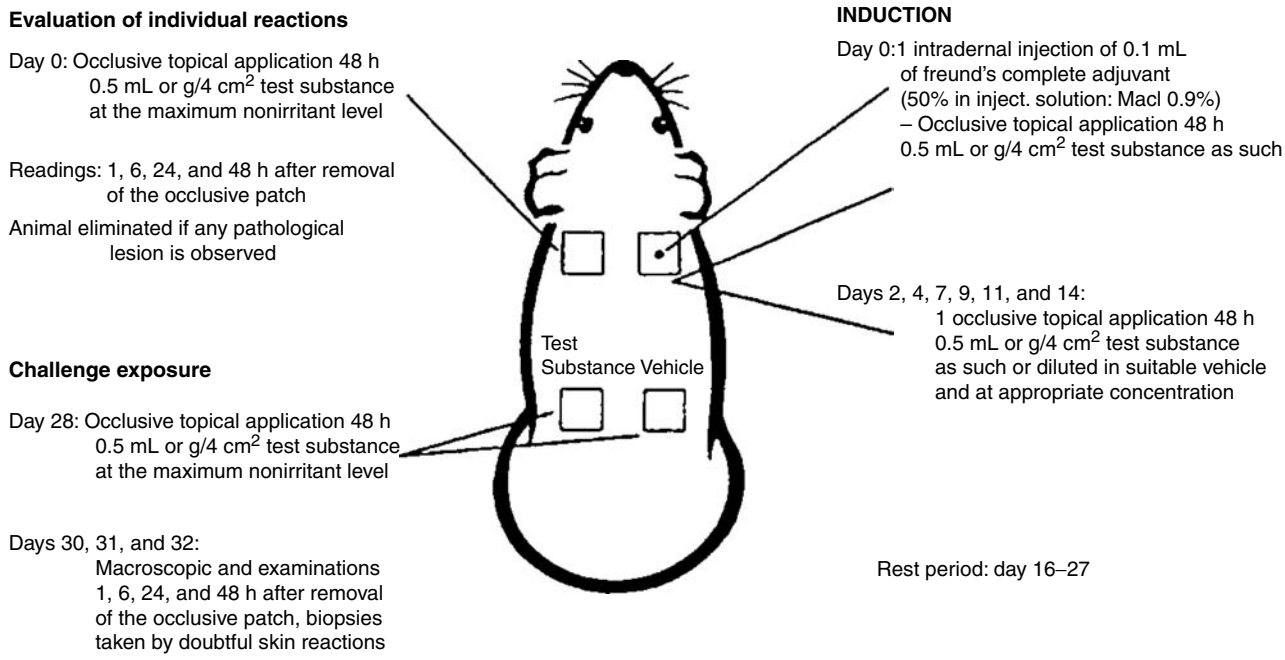


FIGURE 52.12 Epicutaneous maximization test. (From Guillot, J.P. and Gonnet, J.F., *Curr. Probl. Dermatol.*, 14, 220–247, 1985.)

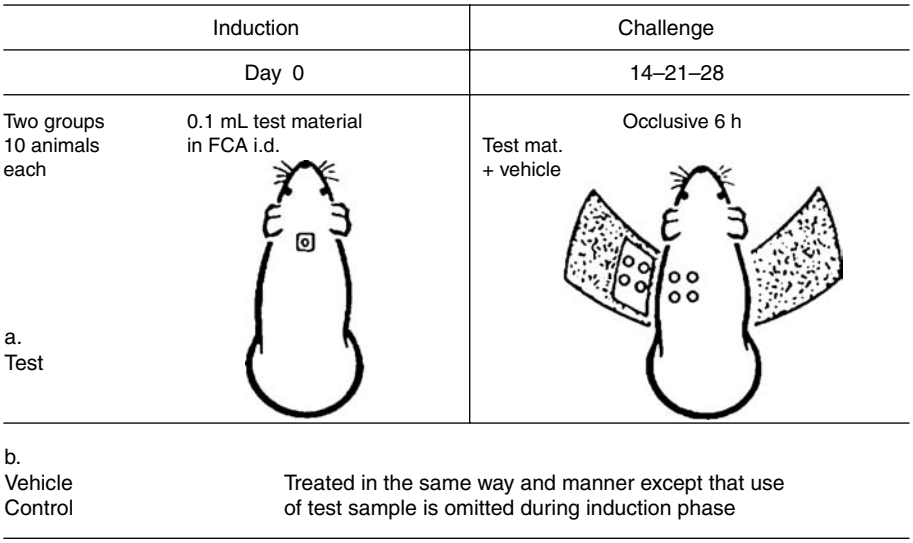


FIGURE 52.13 Single-injection adjuvant test.

Skin responses to challenge test are scored according to a standard grading scale. Histological examinations are performed in cases where skin lesions occur or reactions are doubtful.

Biopsies are carried out about 6 h after the first reading or immediately after subsequent readings. Excised skin specimens are fixed and embedded in paraffin, and sections are stained with hematoxylin and eosin. No control animals are involved in this assay.

Authors reported (Rochas et al., 1977; Guillot and Gonnet, 1985) that in their hands the test was sufficiently sensitive to identify end-use products or their components that show even a slight allergenic potential.

52.13 SINGLE-INJECTION ADJUVANT TEST

In the eyes of the originators (Goodwin et al., 1981), the single-injection adjuvant test (SIAT) procedure is simple to perform, and represents a suitable alternative to the GPMT of Magnusson and Kligman (1970). This technique is a modification of the methodology used by Connor et al. (1975) for allergenicity studies with sultone sensitizers in alkyl ether sulfates. Two groups of 10 guinea pigs each are used (Figure 52.13).

For the induction phase, the dissolved or suspended test article in a minimal quantity of suitable solvent is mixed with FCA to the chosen "final" induction concentration, determined in a preliminary irritation test, causing not

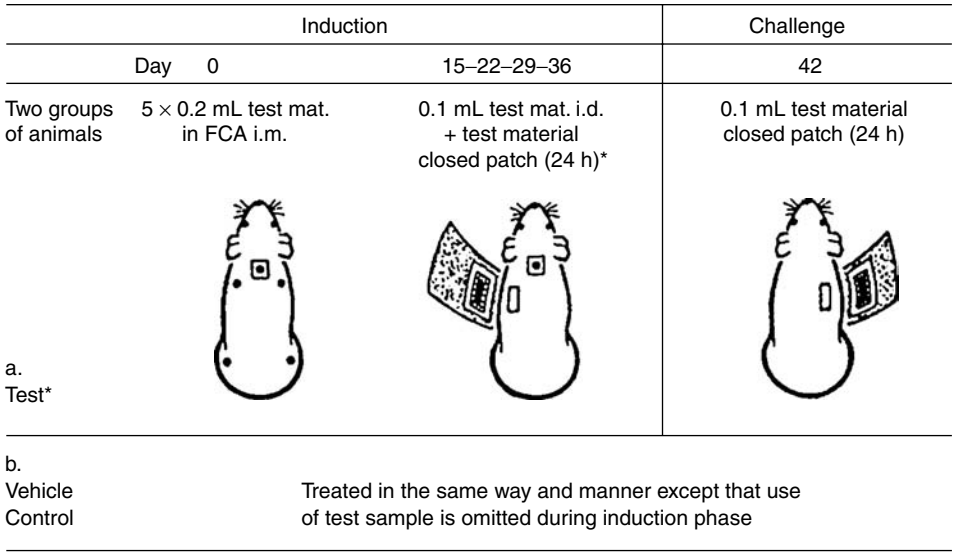


FIGURE 52.14 TINA test.

more than moderate skin irritation. This mixture is injected intradermally at a dose of 0.1 mL between the animal's shoulders.

The challenge test is performed on day 14. Filter paper in aluminum patch test cups or Finn chambers or other type of "patch unit" is saturated with primary nonirritating concentration(s) of the test article and applied occlusively for 6 h to a shaved skin site on one animal flank. Subsequent rechallenge(s) may be performed at weekly intervals, necessitating the use of four new control animals. Controls are treated similar to test animals, except that during induction the test article is omitted.

The skin test sites are examined approximately 22 and 46 h after removal of the patch, and the elicited skin reactions are estimated according to a standard scoring system.

The sensitivity of the SIAT can be improved by increasing the number of intradermal exposures during the induction phase (DIAT or TIAT, i.e., double or triple induction injection), or by rechallenging (booster effect).

Even if less sensitive than the GPMT of Magnusson, the SLAT is easy to perform, more flexible, and not as time-consuming. Other merits and deficiencies are discussed by Goodwin et al. (1983), Goodwin and Johnson (1985), and Basketter and Allenby (1991). SIAT is not suitable for testing nonsoluble or suspensible chemicals and end-use products.

52.14 THE TIEREXPERIMENTELLER NACHWEIS (TINA) TEST

This test protocol involves 25 test and 10 control guinea pigs (Figure 52.14). The induction procedure requires intramuscular and intradermal administration, as well as a cloth patch test to apply the test sample to the animal (Ziegler, 1976, 1977; Ziegler and Süss, 1985).

At day 0, a mixture of FCA and the test article at a dose of 0.2 mL and at a slightly irritant concentration is administered intramuscularly into all four extremities and into the clipped neck region of the test animal. On day 14 the neck region and one flank of the animals are shaved. Then 10% SLS in Vaseline or 70% dimethyl sulfoxide (DMSO) is applied to the flank skin. After 24 h, 0.1 mL of the test sample at a minimal irritant concentration is administered intradermally into the neck region. Simultaneously, the flank skin is exposed to the test sample at a slightly irritant concentration, using a 24-h occlusive patch. On day 17 the patch is removed. This double exposure to the corresponding skin sites is repeated four times at weekly intervals.

On day 42, the right flank of all experimental animals is shaved for challenging. The challenge test is performed on the shaved contralateral flank on day 42 using a 24-h occlusive patch test with the test article at a nonirritant concentration in suitable vehicle. Readings of skin responses and their grading are performed on four subsequent days. Skin reactions with a duration of 48 h and more are considered positive.

Control animals are treated similar to test animals, except that the use of the test article is omitted during the induction phase. Rechallenge or cross-test may follow at intervals of 7–14 days.

The TINA test is a sensitive but time-consuming procedure and is not suitable for testing poorly soluble or insoluble test articles, semisolids, or end-use products. The database is new.

52.15 THE FOOTPAD TEST

Two groups of 10 guinea pigs each are used (OECD, 1992) for this test (Figure 52.15).

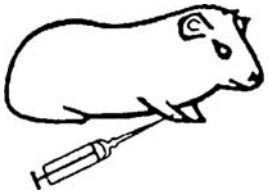
	Induction	Challenge
	Day 0	21
Two groups 10 animals each	Footpad injection: 0.05 mL 1% sol. in FCA	0.3 mL e.c. 1% solution in solvent system of guinea pig fat: dioxan: aceton, 1: 2: 7.
a. Test		
b. Vehicle Control	Treated in the same way and manner except that use of test sample is omitted during induction phase	

FIGURE 52.15 Footpad test.

For induction, on day 0 a 1% mixture or suspension (w/v) of the test article in complete Freund's adjuvant is administered at a dose of 0.05 mL into the front footpad of the test animals. The challenge test is performed on day 7. The test article usually is dissolved at a concentration of 1% (primary nonirritating one) or less in a vehicle formulated from guinea pig fat: dioxone: acetone (1:2:7) and applied open epicutaneously to the clipped flank skin at a dose of 0.3 mL to a circular test site of 8 cm^2. About 24 h after challenge, the test area is depilated and washed off with lukewarm water (37°C). Approximately 3 h after depilation, examination and grading of the elicited skin response are done according to a standard rating scale. Rating of these skin reactions is repeated 24 h later.

Control animals are treated in the same way as test animals, except that the use of the test article during induction is omitted.

The footpad test may be modified by using intradermal administration of the test article at primary nonirritating concentration in bidistilled water or in saline for challenging. Limits and deficiencies of this animal assay are similar to those of the Draize test. The database of this animal assay is poor.

52.16 THE GUINEA PIG ALLERGY TEST ADAPTED TO COSMETIC INGREDIENTS

To evaluate the safety of different types of cosmetic formulations, toiletries, and their individual components, the authors developed a testing methodology based on the combination of two protocols (Dossou et al., 1985).

Three groups of 12 or 24 guinea pigs each are used: one group for topical application, one for intradermal administration of a test article, and one control group. If indicated, additional vehicle groups can be added. After each epicutaneous application, animals are placed into individual restrainers for 2–4 h, allowing no movement and licking.

52.16.1 Protocol I—Induction by Topical Route

On day 0, all animals are treated with 0.2 mL Freund's complete adjuvant, administered into the footpad of one of the animal's hind legs, which has been cleaned with ethanol. An area of 3 × 5 cm^2 on the scapular region is shaved (hair product testing) and depilated (skin product testing) on days 0, 2, and 4, about 6 h prior to open application of a dose of 0.5 mL of the neat test article or its dilutions or suspensions in suitable vehicle to this test site. If appropriate (weaker contact allergen), the total number of induction exposures can be increased up to nine (Figure 52.16).

52.16.2 Protocol II—Induction by Injection

The test animal group is treated by administration of a 1:1 mixture of FCA/dissolved test article or 1:1 emulsion of (FCA + unsoluble test article)/bidistilled water into the footpad of one of the animal's hind legs (Figure 52.17).

For the challenge, after a rest period of 11 days, all experimental animals are challenged by open application of 10 µL of one or more test samples, which are not skin irritating, to the clipped and depilated back and lumbar region. The elicited reactions are read 24 and 48 h after challenging and the intensity is scored. Both protocols can be modified by rinsing the test article, increasing the frequency of exposure during induction, and involving cross-tests or rechallenge tests. The control group is challenged in the same way as the test group, except that the use of the test article is omitted during induction.

The two protocols are complementary and linked. The topical route protocol simulates the conditions of anticipated exposure to end-use products, while the intradermal route is as exaggerated and sensitive as the GPMT. This assay is simple to perform, variable, sensitive, and suitable for testing dermatologics, cosmetics and toiletries, as well as single chemicals, but during immobilization it is stressful for the animals. The database is new.

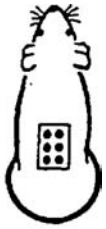
	Induction		Challenge
	Day 0	2–4	15
Three groups 8 animals each	0.2 mL FCA footpad test material 0.5 mL/15 cm^2 e.c.	Test material 0.5 mL/15 cm^2 e.c.	Test material 0.01 mL e.c.
a. Test			
b. Vehicle Control	Treated in the same way and manner except that use of test sample is omitted during induction phase		
c. FCA Control	During induction treated with FCA i.d. exclusively and challenged		

FIGURE 52.16 Guinea pig allergy test (1).

	Induction		Challenge
	Day 0–3	4	15
Two groups 8 animals each	No treatment	0.2 mL FCA + test material footpad inject.	Test material 0.01 mL e.c.
a. Test			
b. Vehicle Control	Treated in the same way and manner except that use of test sample is omitted during induction phase		

FIGURE 52.17 Guinea pig allergy test (2).

52.17 THE EAR/FLANK TEST (STEVENS TEST)

This animal assay (Figure 52.18) is recommended as useful for rapid sensitization potential screening of a wide range of industrial chemicals by Stevens (1967). The use of the ears for induction of sensitization has been described by Davies (1964). Two groups of 10 guinea pigs each are used.

For the induction phase, the test article, diluted in suitable vehicle and at an appropriate concentration, is applied once daily on 3 consecutive days to the outer surface of the ears at a dose of 0.1 mL/ear.

For challenging, on day 7 a range of primary nonirritant concentrations of test article in suitable vehicle and at a dose of 0.2 mL are applied to a clipped flank of 10 test animals on a marked circular area with a diameter of 1.0 cm. Control animals are challenged. Elicited skin reactions are graded 24 h later according to a standard rating scale. Rechallenge or cross-test may follow at intervals of 1–2 weeks on contralateral flanks.

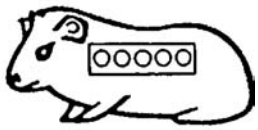
	Induction	Challenge
	Day 0–1–2	7
Two groups 10 animals each	0.1 mL/ear e.c.	0.2 mL/cm² e.c.
a. Test		
b. Vehicle Control	Treated in the same way and manner except that use of test sample is omitted during induction phase	

FIGURE 52.18 Ear flank test (Stevens test).

There are four advantages of the ear/flank test:

1. No clipping or shaving of the outer surface of the ears is required before treatment.
2. The flanks are virgin skin test sites.
3. Test material applied to the ear appears not to be interfered with by the guinea pig, and thus the use of occlusive dosing can be avoided.
4. It is suitable for testing of final or end-use products.

The classification system is not intended to imply that this animal assay fails to detect weak sensitizers. However, even a negative result in the ear/flank test may be utilizable in predicting that an industrial product is not a strong contact sensitizer. The database is relatively slim.

52.18 CONCLUSIONS

The most promising preventive measure for reducing the incidence of allergic contact dermatitis in a population is to limit the contamination of our environment with potential contact allergens (Department of Health, Education, and Welfare, 1975).

A skilled, selected, and properly performed animal assay on guinea pigs offers a useful tool to assess the sensitizing potential of individual chemicals as well as end-use products (Andersen and Maibach, 1985a,b). This becomes increasingly important as more predictive animal data become available for which a good correlation exists with epidemiological findings. It is of paramount importance in safety assessment that the difference between animal skin sensitizing risk and human health hazard is clearly stated. Thus, for the purpose of hazard evaluation, a positive result originating from animal sensitization assay does not necessarily mean that a hazard for humans exists, taking assumed exposure conditions to environmental contactants into consideration.

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53 Test Methods for Allergic Contact Dermatitis in Humans

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53.1 INTRODUCTION

Allergic contact dermatitis (ACD) is a commonly occurring inflammatory skin disease that appears as a delayed skin response following skin contact with an allergenic chemical. It is characterized by erythema, edema, and vesiculation. Regulatory agencies such as the Food and Drug Administration (FDA), U.S. Environmental Protection Agency (EPA), and Consumer Product Safety Commission (CPSC) often require that chemicals and untested substances that are intended to be newly introduced into the marketplace be evaluated for this hazard potential.

ACD has widespread occurrence, in part because of the annual introduction of large numbers of new chemicals into the marketplace, some of which are ultimately found to be allergenic under use conditions. In addition, older allergenic chemicals employed in occupational settings provide a continuing source of skin disease and are difficult to eliminate from the environment.

Medicaments and cosmetics contain preservatives and fragrances that may be allergenic (Bandmann et al., 1972; Marzulli and Maibach, 1980; Opdyke, 1974). A more detailed list of allergenic substances would include rubber, plastics, metals, epoxy resins, wood products, metal-working fluids, printing chemicals, and others (Maibach, 1987). Culinary and nonedible plants constitute another sizable source of allergenic chemicals (Fisher, 1986).

The *American Journal of Contact Allergy* (now called *Dermatitis*), started in 1989, was introduced as the official journal of the American Contact Dermatitis Society. It and the older nondomestic journal *Contact Dermatitis* are the current principal sources of information about ACD. *Dermatosen's* articles in German and English provide many scholarly presentations.

Although ACD closely resembles irritant contact dermatitis on gross inspection of the skin, ACD has an immunologic etiology that is lacking in irritant dermatitis. Accordingly, tests for ACD potential must demonstrate that the chemical is capable of producing a more severe subsequent skin effect than was encountered on initial contact, signifying an allergic (altered) response rather than an irritant response. Conversely, skin irritation as a cause for skin effects must be excluded. That is, a positive skin response at challenge must be produced by a clearly nonirritant concentration of the test substance.

Animal tests are often preferred as compared with human tests for predicting human ACD potential. This is a precautionary measure that is undertaken to avoid sensitizing a significant segment of the human population to the test chemical and to a vast array of closely related (cross-reacting) chemicals that will be encountered by the test subject at a later time.

Nonanimal *in vitro* alternative tests are currently under investigation but none has been validated to date.

Human tests for ACD potential are often needed as follow-up to animal tests, since the correlation between animal and human test results is not exact (Marzulli and Maguire, 1982). Human tests are employed both for forecasting ACD potential of new chemicals and for diagnosing ACD in clinical patients that present themselves to investigative dermatologists for evaluation and treatment of contact dermatitis.

Allergenic chemicals are sometimes purposefully introduced into the marketplace if there is some medical benefit to be achieved, and the therapeutic index suggests that the concentration required for efficacy is below that likely to result in sensitization of large number of users. Benzoyl peroxide is allergenic and falls into this category. It is marketed as a drug for treating acne vulgaris, with a warning label to discontinue use if ACD occurs. Others, like formaldehyde, have a potential for sensitizing, but may be well tolerated at low concentrations and appropriate use concentrations.

53.2 PREDICTIVE TESTS

A large database exists involving animals and humans that have been tested with a wide variety of chemicals for skin

TABLE 53.1
Predictive Tests for Skin Sensitization Humans

Test	Number of Subjects	Amount or Concentration of Test Substance	Vehicle	Skin Site	Type Patch	Induction			Challenge	Reference
						Number of Patches	Duration	Rest		
Schwartz	200	Fabric			Fabric	1	5 days	10 days	48 h patch; observe 10 days	Schwartz (1941)
Schwartz	200	1 in. fabric, liquid, or powder		Arm, thigh, or back	Cellophane covered with 2 × 2 in. elastoplast	1	72 h	7–10 days	72 h; same site; observe 3 days	Schwartz (1960)
"Prophetic" Schwartz–Peck	200	1/4 in.^{2} 4-ply gauze, liquid saturated[a]	Petrolatum or corn oil	Arm or back	1 in.^{2} nonwaterproof cellophane covered with 2 in.^{2} adhesive plaster	1	24 h or 3 or 4 days	10–14 days	48 h; any site especially thin keratin; observe 3 days; compare new and old formulas	Schwartz and Peck (1944), Schwartz (1951)
"Repeated insult" Shelanski	200	Proportional to area of ultimate use	Mineral oil		Occlusion; follows Schwartz test	10–15	24 h every other day; same site	2–3 weeks	48 h patch	Shelanski (1951), Shelanski and Shelanski (1953)
"Repeated insult" Draize	100 males 100 females	0.5 mL or 0.5 g		Arm or back	1 in.^{2}	10	24 h alternate days	10–14 days	Repeat patch on new site	Draize et al. (1944)., Shehinski (1951), Draize (1959)
Modified Draize	200	0.5 mL or 0.5 g (high concentration)	Petrolatum	Arm	Square BandAid, no perforations	10	48 h	2 weeks	Patch on new site 72 h with nonirritant concentration	Marzulli and Maibach (1973, 1974)
"Maximization" Kligman	25	1 mL 5% SLS[b] followed by 1 mL 25% test material	Petrolatum	Forearm or calf	1.5 in.^{2} Webril occluded with Blenderm; held in place with perforated plastic tape	5 (same site)	24 h SLS followed by 48-h test material for each of five inducing applications	10 days	1 in.^{2} patch on lower back or forearm; 0.4 mL of 10% SLS for 1 h followed in 24 h by 0.4 mL of 10% test material for 48 h	Kligman (1966a)
Modified "maximization"	25	Same as maximization				7	24 h SLS followed by 48-h test material for each of seven inducing applications; no patch for 24 h between each of seven inducing applications	10 days	2% SLS for 1/2 h followed by 48-h patch with test material	Kligman and Epstein (1975)

[a] Modified for solids, powders, ointments, and cosmetics. Concentration, amount, area, and site of application are considered important in evaluating results. Authors recommended that cosmetics be tested uncovered.

[b] Sodium lauryl sulfate (SLS) pretreatment is used to produce moderate inflammation of the skin. SLS is mixed with test material when compatible. SLS is eliminated when the test material is a strong irritant.

sensitization potential. These data are available for study of the relationship of chemical structure and potency as skin sensitizers. Dupuis and Benezra (1983) and Benezra et al. (1989) summarized chemical properties that appeared to be associated with a propensity for skin sensitization. More recently, structure–activity relationships were investigated by Ferguson et al. (1994). This group has targeted electrophilicity as an important factor in a chemical's capacity to sensitize. Accordingly, they have employed measures of electrophilicity to predict sensitization potential.

Human test methods for forecasting skin sensitization potential were largely developed and refined during the period 1941–1975 and are summarized in Table 53.1. Currently, the modified maximization technique of Kligman and Epstein (1975) and the modified Draize procedure (Table 53.1) are methods of choice.

In these human studies, the test chemical is applied to the skin under an occlusive patch. This chemical trauma is repeated 7–10 times over a 3-week period to induce sensitization of the test chemical. This is followed by a rest period of 10 days to 2 weeks. The skin is then challenged (to see if an immune-based altered response has taken place) using a new skin site and application of a nonirritant concentration of the test material under occlusion. Induction may be accomplished by employing enhancing techniques such as repetition of the chemical insult (Shelanski, 1951), using a high concentration of test material at induction (Marzulli and Maibach, 1973, 1974), treating the skin with sodium lauryl sulfate (SLS) (Kligman, 1966b), skin stripping (Spier and Sixt, 1955), or freezing (Epstein et al., 1963). Freund's adjuvant (Freund, 1951) was among the earliest techniques to improve the sensitization response in animal tests.

The size of the test population is important with regard to interpretation of findings. Henderson and Riley (1945) discussed how the total number of test participants employed may affect the predictive accuracy of the data obtained, when analyzed statistically. Careful consideration must be given to two aspects:

1. The sample size of test subjects must be large enough so that results are valid for the population at large, yet small enough to be logistically feasible in the laboratory.
2. The laboratory test must have the capacity to predict likelihood of occurrence under use conditions.

53.3 DIAGNOSTIC TESTS

In diagnostic tests, a preparation is applied to a clinical patient's skin under an occlusive patch for 48 h and the skin is evaluated for evidence of erythema, edema, or more severe skin changes occurring 24, 48, or 72 h after removal of the patch. Allergenic materials are thereby identified by producing skin disease on a small scale with offending chemicals.

The American Academy of Dermatology has worked collaboratively with Hermal Pharmaceutical Laboratories, Inc., New York. This organization can provide further details regarding diagnostic test methods and types and sources of test allergens. A booklet by James G. Marks and Vincent A. DeLeo (1992) entitled *Patch Testing for Contact and Occupational Dermatology* is available from Hermal Laboratories.

The North American Contact Dermatitis Group, a task force of the American Academy of Dermatology, consisting of a dozen or so dermatologist specialists, focuses attention on allergic contact dermatitis. They review test methods, standardize techniques, collect information relating to the ACD potential of marketplace products and chemicals, and publish their findings.

One of the more recent refinements in interpretation of diagnostic test results consists of an assessment of the relevance of positive patch-test findings to the diagnosis. The investigator must first establish that positive patch-test results are consistent with a history of exposure to a particular chemical in a product and must exclude other possible environmental exposure conditions. Next, the location of the present dermatitis must correspond to the site of contact with the putative offending chemical. Finally, the patch-test concentration must be nonirritating, as can be demonstrated by a dose–response effect when dilution of the putative allergen is employed. An operational definition of ACD has been suggested (Ale and Maibach, 1995) using the following algorithm to establish the relation between a positive patch test and the likelihood of clinical ACD: (1) history of exposure, (2) appropriate morphology, (3) positive patch test to a nonirritating concentration of the putative allergen, (4) repeat patch test if excited skin syndrome is operative (more than one positive patch test), (5) employ serial dilution patch testing to distinguish allergen from marginal irritant, (6) employ use test or open patch test, and (7) resolution of dermatitis.

An example of test results obtained by the North American Contact Dermatitis Group was reported by Storrs et al. (1989) and is given in Table 53.2. The most common sensitizers identified were nickel, *p*-phenylenediamine, quaternium-15, neomycin, thimerosol, formaldehyde, cinnamic aldehyde, ethylenediamine, potassium dichromate, and thiuram mix. Ten participating investigation centers were involved, including five university clinics, two large multispecialty clinics, and three private offices.

53.4 EXCITED SKIN SYNDROME

"Angry back" and "excited skin" are terms used to describe a hyperirritable skin condition that occurs when multiple concomitant inflammatory skin conditions prevail. When this hypersensitive skin condition exists, false positive test results may occur. Details and strategies for dealing with the situation are discussed by Bruynzeel and Maibach (1991).

TABLE 53.2
Results of Tests on 1119 Patients with Suspected Allergic Contact Dermatitis, Using 32 "Standard Allergens"

	Interpretation		Relevance			
Allergens[a]	**Total tallied**	**Allergic**	**Doubtful**	**Irritant**	**Present**	**Past**	**Relevance (%)**
Balsam of Peru 25% petrolatum	1122	37	13	2	10	3	35
Benzocaine 5% petrolatum	1135	40	3	0	18	6	60
Benzoyl peroxide 1% petrolatum	1115	20	6	3	6	2	40
Black rubber paraphenylenediamine mix (PPD mix 0.6% petrolatum)[b]	1140	16	1	3	10	1	69
Caine mix less benzocaine 3% petrolatum[c]	1117	22	7	8	7	4	50
Carba mix 3% petrolatum (carba rubber mix)[d]	1135	38	14	6	23	4	71
Cinnamic alcohol 5% petrolatum	1046	28	4	4	13	2	54
Cinnamic aldehyde 2% petrolatum	1048	62	21	60	22	5	44
Dibucaine 1% petrolatum	1009	8	1	2	2	1	38
Cyclomethycaine sulfate 1% petrolatum	1012	8	I	4	2	0	25
Epoxy resin 1% petrolatum	1129	21	3	0	9	3	57
Ethylenediamine dihydrochloride 1% petrolatum	1120	66	2	0	28	18	70
Eugenol 4% petrolatum	1016	14	3	5	3	3	43
Formaldehyde 2% water	1144	70	9	13	27	5	46
Hydroxycitronellal 4% petrolatum	1049	16	2	2	6	2	50
Imidazolidinyl urea 2% water	1134	17	5	2	5	0	29
Isoeugenol 4% petrolatum	1012	24	5	1	5	3	34
Lanolin alcohol (wool wax alcohol) 30% petrolatum	1135	14	5	1	10	1	79
Mercapto mix 1% petrolatum (mercapto rubber mix)[e]	1132	30	3	0	21	3	80
Mercaptobenzothiazole 1% petrolatum	1141	33	4	2	21	4	76
Neomycin sulfate 20% petrolatum	1131	75	5	2	25	19	59
Nickel sulfate 2.5% petrolatum	1123	109	7	8	40	43	76
Oak moss 5% petrolatum	1038	20	3	0	7	4	55
para-Tertiary-butylphenol formaldehyde resin 1% petrolatum	1129	9	0	3	5	2	78
Potassium dichromate 0.5% petrolatum	1138	59	15	34	19	7	44
p-Phenylenediamine 1% petrolatum	1138	79	6	2	33	14	59
Quaternium-15 2% petrolatum	1129	76	1	0	37	6	57
Quaternium-15 2% water	1103	43	12	0	25	0	58
Rosin (colophony) 20% petrolatum	1132	22	4	0	8	1	41
Tetracaine 1% petrolatum	1014	8	1	2	1	3	50
Thimerosal 0.1% petrolatum	1137	70	10	4	15	20	50
Thiuram mix 1% petrolatum (thiuram rubber mix)[f]	1137	44	8	0	25	5	68

[a] Not every patient was tested with every allergen.

[b] *N*-phenyl-*N*′-cyctohexyl-*p*-phenylenediamine 0.25%, *N*-isopropyl-*N*′-phenyl-*p*-phenylenediamine 0.10%, *N*,*N*′-diphenyl-*p*-*p*~phenylenediamine 0.25%.

[c] Dibucaine 1%, tetracaine 1%, cyclomethycaine sulfate 1%.

[d] Diphenylguanidine 1.0%, zinc diethyldithiocarbamate 1.0%, zinc diethyldithiocarbamate 1.0%.

[e] *N*-cyclohexyl-2-benzothiazolesulfenamide 0.333%, 2.2′-benzothiazyl disulfide 0.333%, 4-morpholinyl-2-benzothiazyl disulfide 0.333%.

[f] Tetramethylthiuram disulfide 0.25%, tetramethylthiuram monosulfide 0.25%, tetramethylthiuram disulfide 0.25%, dipentamethylenethiuram disulfide 0.25%.

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54 Allergic Contact Dermatitis: Elicitation Thresholds of Potent Allergens in Humans

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54.1 INTRODUCTION

Contact dermatitis is an inflammatory condition caused by direct skin exposure to an offending chemical with or without a requirement for ultraviolet light. There are two distinct types of contact dermatitis: irritant contact dermatitis (ICD) and allergic contact dermatitis (ACD). ACD is an eczematous disease mediated through immune mechanisms. It is an acquired skin disorder that occurs at sites of contact with small chemical haptens in only those individuals who have been previously exposed to, and immunologically sensitized to a particular chemical. In contrast to ICD, only a small percentage of the population develops an eruption when exposed to chemicals causing ACD. The most common chemical allergens causing the condition in North America include nickel sulfate, as well as the pentadecylcatechols, the active moiety in plants of the *Rhus* family, which include poison ivy, poison oak, and poison sumac [1].

An extensive literature discusses weak versus strong allergens. The term refers either to the percent of population sensitized or to molecular potency—the concentration of a chemical eliciting ACD. For the purposes of this chapter, strong allergens elicit a reaction at low concentration; weak allergens require a higher dose. This study creates a database of xenobiotics that have been described as human allergens of extreme molecular potency, eliciting reactions at low challenge concentrations. The resulting biologically based algorithm derived from analysis of the difference in structural alerts statistically associated with protein reactivity between the two categories (evaluating quantitative structure–activity relationship, QSAR), may permit *a priori* classification of untested chemicals as strong or weak allergens.

The database of chemical structures known to cause ACD in humans that are classified by potency is limited, compared with more than a thousand compounds considered as allergens in animals [2]. Of those, allergens of extreme potency number only a few dozens. This might be due to the fact that most dermatologists routinely test patients using standard patch-test series only at the single concentration recommended, which leads to an undifferentiated "yes"/"no" diagnosis for hypersensitivity. It may also be, however, that allergens that meet our criterion of "extreme" are uncommon. It is intuitive but not documented that with equal skin exposure, a potent (in ppm) allergen might be expected to sensitize more individuals than a less potent allergen.

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54.2 MATERIALS AND METHODS

As the terms utilized here (potency, ppm, etc.) are not typically indexed, standard dermatological journals were searched manually as well as through MEDLINE, from 1956 to the present. Manually searched journals included the following: *Contact Dermatitis, American Journal of Contact Dermatitis, British Journal of Dermatology, Journal of American Academy of Dermatology, Archives of Dermatology, Acta Dermato-Venereologica, Acta Dermato-Venereologica (Supplements), Journal of Investigative Dermatology.*

Only human data were utilized. Standard patch testing, provocative use test, and repetitive open application test were used in studies reviewed in our manuscript. Keywords included human, ACD, elicitation, patch test, threshold concentration, metals, biocides. References from retrieved papers were examined. Chemicals were included based on the following criteria:

a. Arbitrary maximum elicitation concentrations of 500 ppm (0.05%)
b. Defined chemical structure
c. A minimum record of two patients or volunteers to any given compound

We do not differentiate between ACD (allergic dermatitis from relevant exposure) and contact allergy (a positive patch test of uncertain clinical relevance) because the literature used in our manuscript does not generally permit such distinction.

54.3 RESULTS

54.3.1 Metals

54.3.1.1 Mercury

Mercury was recognized as a contact sensitizer in 1895. Exposure can occur with three different chemical forms: metallic mercury such as yellow oxide of mercury or mercury from a broken thermometer, mercury salts (phenylmercuric salts), such as for example, in tattoos containing the red pigment cinnabar (mercuric sulfate), and organic mercury including thimerosal, methylmercury, and merbromin. The most common contact with mercury is through thimerosal, which is used as an antiseptic and preservative. Thimerosal may be found in topical medications, especially ophthalmic and nasal preparations, cosmetics, and as a preservative in vaccines. Methylmercury exposure occurs through consumption consume of the contaminated fish.

The *American Journal of Contact Dermatitis* identified thimerosal as the contact allergen (nonallergen) of the year in 2002. Although it is the fifth most frequently found contact allergen in patch-tested patients in the United States, the 1998–2000 NACDG (North American Contact Dermatitis Group) database reported thimerosal to have a definite or probable relevance in only 2.9% of the patients with a positive test. Because of this very low relevance frequency, the NACDG has elected to delete thimerosal from its allergen testing "standard tray" of patch-test allergens; however, it is still part of the T.R.U.E. TEST patch-test series.

Whereas avoidance of thimerosal-containing vaccines in patients with contact hypersensitivity may be considered, adverse effects other than a localized injection site reaction are rare. In patients with symptomatic oral disease adjacent to mercury amalgams, patch tests with a dental series containing metallic mercury allergens may be appropriate [3].

In the human repeat-insult patch test, mercuric chloride caused 92% positive reactions, making the ionized form a class 5 (extreme) sensitizer on the Magnusson–Kligman scale [4]. Metallic mercury (e.g., in amalgam), is only a moderate topical sensitizer.

Tested with mercuric chloride ($HgCl_2$), 0.05% (500 ppm; exposure time of 2 days, reading on day 3), 58 of 377 patients reacted; the number of positive reactions was significantly greater among patients with pierced ear lobes (29/107) than among those who did not have pierced ears (27/270) [5]. Patients with baboon syndrome (systemic eczematous contact-type dermatitis) and gold dermatitis due to ear lobe piercing were tested with 0.05% (500 ppm) mercuric chloride (patch tests applied for 2 days, reading on day 3); five of five patients with baboon syndrome were patch-test positive. Twenty-one of thirty-five patients reacted positively to patch testing with mercury pierced ears [6].

In 13 mercury-allergic patients, the mean threshold concentration of mercury has been evaluated in two different vehicles: distilled water and petrolatum.

- Mercuric chloride 0.05% (500 ppm) in distilled water: patch tests were positive in two patients and negative in petrolatum.
- Mercuric chloride 0.025% (250 ppm) in distilled water: patch tests were positive in three patients and in four patients in petrolatum.
- Mercuric chloride 0.0031% (31 ppm) in petrolatum: patch tests were positive in two patients and negative in distilled water.
- Mercuric chloride 0.0015% (15 ppm) in distilled water: patch tests were positive in three patients and in two patients in petrolatum [7].

The permissible 8-hour Time-Weighed Average exposure (TWA) level of mercury vapor in the workplace is 0.05 mg/m^3 [8]. Investigation of mercury sensitivity among health professionals revealed a sensitization rate of 2.4–7.2% [9].

In two cases of occupational exposure to mercury vapors at a level of 9.9 mg/m^3, medical professionals presenting with clinical symptoms of systemic sensitization reacted to challenge testing with 0.05% (500 ppm) aq. mercuric chloride, one of them to 0.05% (500 ppm) thimerosal in petrolatum [10]. When 12 patients with oral mucosal lesions associated with amalgam restorations were patch tested with aq. $HgCl_2$ 0.05% (500 ppm), five gave a positive skin reaction (patch tests applied for 2 days, read on day 3) [11].

Patients with oral mucosal lichenoid lesions due to amalgam restorative materials were tested with several organic

mercury compounds. Reactions were read at 24 and 48 h, and also after 3, 10, and 17 days. Of 19 patients, tests with 0.05% (500 ppm) phenylmercury acetate gave six positive reactions after 3 days, one on day 10 and one on day 17. The test with 0.05% (500 ppm) phenyl mercury nitrate was positive in two patients on day 3 and one on day 10. With 0.05% (500 ppm) thimerosal, one patient each was positive on days 3, 10, and 17 [12]. Of 1025 contact dermatitis patients tested, 215 (21%) were positive to thimerosal overall, 12 (8%) to 0.05% (500 ppm) thimerosal in petrolatum, and 138 patients showed positive reactions to 0.05% (500 ppm) ethylmercury chloride in petrolatum [13].

There is a suggestion of the high frequency of sensitization to thimerosal in atopic children. Of four children tested with serial dilutions of thimerosal in petrolatum (exposure time of 2 days, reading time on days 2 and 3), all showed a positive reaction at 0.1% (1000 ppm) and three also at 0.01% (100 ppm) [14].

The relative risk of sensitization to different classes of allergens was evaluated in a multicenter study involving 31,849 random eczema patients from 24 dermatology departments, including healthcare workers, who were tested over a period of 3 years (1992–1995). Significantly higher sensitization rates overall were recorded among healthcare workers when compared with the control group of patients (not involved in healthcare-related occupations). Incidence of positives to phenyl mercuric acetate (0.01%) patch tests (exposure time 24–48 h, reading time at 72 h) was 4% of 1349 in the medical occupations versus 3.7% of 10,486 controls [15]. Among 10,974 patients tested with phenyl mercuric acetate aq. 101 (1.7%) reacted to a concentration of 100 ppm [16].

A similar multicenter study evaluated sensitization rates to different series of preservatives, antimicrobials, and industrial biocides. Among 19,454 dermatology patients tested with preservatives of the standard series over the period 1990–1994, 4.0% reacted to thiomerosal at 0.05% (500 ppm). Tested with a different preservatives series, 440 of 9361 reacted to 0.05% (500 ppm) thiomerosal and 101 of 1852 to phenyl mercuric acetate [17].

In 24 dermatological departments, 6548 randomly selected patients were tested over a period of 4 years (1990–1994) for allergy to sodium timerfonate. At 0.05% (500 ppm) (exposure 24–48 h, reading at 72 h), 31 had positive reactions [17]. In a series of studies, different cohorts of subjects who had previously given positive patch-test reactions to thiomerosal were tested with ethylmercury chloride in ethanol and aq. methylmercury chloride. Test reading was done on days 2 and 4. 0.0165% (165 ppm) ethylmercury chloride in ethanol resulted in 32 of 32 positives in a first study [17]. In the second study, 36 of 36 patients had positive reactions to 0.031% (310 ppm) aq. methylmercury chloride, 18/18 to 0.031% (310 ppm) ethylmercury chloride in ethanol, and 18/18 to 0.015% (150 ppm) ethylmercury chloride in ethanol [18]. In a third study, 19 of 21 patients had positive reactions to 0.0165% (165 ppm) ethylmercury chloride in ethanol and to 0.031% (310 ppm) aq. methylmercury chloride [19].

54.3.1.2 Gold

Gold can be described as a covert allergen. Since patch testing for gold hypersensitivity has recently become routine in North America, Europe, and Japan, a considerable prevalence of patch-test positivity to gold has been noted. Even so, the degree of clinical relevance of such allergy remains questionable [20], since the condition usually remains "silent" or subclinical [21]. This may explain why few attempts were made to evaluate skin reactivity to low gold concentration in patch tests. Gold sodium thiosulfate (GST) at 0.5% (5000 ppm) in petrolatum is the accepted standard for routine testing.

Defining the clinical relevance of positive gold patch tests is illustrated by the outcome of a patch-test study conducted among an important cohort of clinic patients and volunteers. Patches were removed after 2 days and read after 2 and 4 days. Of 1203, 38 patients were found positive to 0.05% (500 ppm) GST (3.2%), versus 5 of the 105 volunteers (4.8%), most of whom had no identified previous exposure to gold [22]. In a separate study, these authors found 8 patients out of 373 with a positive patch test to 0.05% (500 ppm) GST (2.1%) [23].

In a maximization test program involving human subjects, hypersensitivity was induced by epicutaneous application of 2% gold chloride. On challenge with 0.005% (50 ppm) of the reagent, 16 of 23 gave a positive reaction [4].

54.3.1.3 Nickel

Nickel is recognized as a premier cause of ACD. It belongs to the metal group that reacts with eccrine sweat and can form divalent nickel ions; these, in turn, can penetrate the stratum corneum via the transappendageal or transcellular route to reach the viable epidermis. Reacting there with amino acid residues the resulting nickel-complexed protein may then cause contact allergy [24].

The potential threshold for inducing nickel sensitivity due to contact with irritated skin, also a putative cause of so-called "housewife hand dermatitis," was investigated by a hand-immersion experiment: upon exposure twice daily for 23 days to a surfactant solution, 12 of 20 individuals tested showed positive reactions to 10 ppm aqueous nickel sulfate, 6 of 12 to 5 ppm, 3 of 20 to 1 ppm and 2 of 20 to 0.5 ppm. Also, there was a pronounced difference in reactivities depending on test site [25].

An elicitation concentration of more than 100 ppm nickel (as nickel chloride) has been found necessary to elicit an allergic reaction in a cohort of nickel-sensitized individuals [26].

332 patients with previously diagnosed contact allergy to nickel or a history suggestive of nickel allergy were tested with serial dilutions of nickel sulfate (exposure 24–48 h, reading at 72 h or later) [27].

- Nickel sulfate 0.0005% (5 ppm): patch tests were negative in all patients.
- Nickel sulfate 0.001% (10 ppm): patch tests were positive in 4 of 92 tested patients.
- Nickel sulfate 0.005% (50 ppm): patch tests were positive in 5 of 92.

- Nickel sulfate 0.01% (100 ppm): patch tests were positive in 19 of 329.
- The effect of repeated exposure of the hands to low nickel concentrations over 2 weeks was evaluated by patch testing post exposure in a study of 17 nickel-sensitive volunteers, to simulate occupational exposure [28].
- Nickel chloride 0.02% (200 ppm): patch tests were positive in four patients.
- Nickel chloride 0.01% (100 ppm): patch tests were positive in four patients.

The suggestion is made that a more differentiated approach using dilution series is advisable for diagnostic purposes, rather than the current practice of applying the single 5% nickel sulfate patch test.

The reaction threshold to nickel sulfate was tested in 53 patients sensitized to nickel by patch testing with serial dilutions, both aqueous and in petrolatum. Reactivity was compared between cohorts sensitive to nickel (Ni) only, and an equal cohort sensitized to both nickel and cobalt (Co) [29].

Threshold concentration Ni sulfate 0.039% (390 ppm) aq.: patch tests were positive in two patients sensitive to Ni only, one patient was sensitive to Ni and Co

- Nickel sulfate 0.039% (390 ppm) petrolatum: patch tests were positive in three patients sensitive to Ni only, five patients sensitive to Ni an Co.
- Nickel sulfate <0.039% (<390 ppm) aq.: patch tests were positive in five patients sensitive to Ni only, four patients sensitive to Ni an Co.
- Nickel sulfate <0.039% (<390 ppm) petrolatum: patch tests were positive in one patient sensitive to Ni only, two patients sensitive to Ni an Co.

We conclude the mean reaction threshold for nickel sulfate in water was lower (0.43%) than in petrolatum (0.51%). The lowest thresholds were observed in patients simultaneously sensitive to both nickel and cobalt.

25 nickel-sensitive patients were patch tested by application of a dilution series of nickel sulfate [30]. 112 ppm nickel (0.05% $NiSO_4$) caused reactions in nine patients, 1.12 ppm in one of the patients tested.

The thresholds of sensitivity in individuals with positive reactions to nickel were determined in a serial dilution test with nickel sulfate in petrolatum: of 35 tested, four individuals reacted to 390 ppm, to 190 ppm, and one to 100 ppm [31].

Repeated patch testing with nickel sulfate at 0.0032% (32 ppm) was performed on the upper part of the back of 15 females. Tests were applied for 2 days and read on day 3 after application. Four reacted positively [32].

54.3.1.4 Cobalt

While cobalt is an essential trace element, its salts can pose significant dermatological problems, primarily in the work environment, due to their allergenic potential. Hypersensitivity to cobalt is mostly associated with nickel hypersensitivity, because in metallurgy the two metals are often used in tandem; also due to their chemical similarity and problems in obtaining test materials free of nickel, patch testing for cobalt sensitization often leads to false-positive diagnoses.

Patch testing of 60 cobalt-allergic patients (including cobalt-sensitive only, cobalt- and nickel-sensitive, cobalt- and chromium-sensitive) gave the following results:

- Cobalt chloride 0.0312% (312 ppm) patch tests induced eight positive reactions in distilled water and eight positive reactions in petrolatum.
- Cobalt chloride 0.0156% (156 ppm) patch tests induced one positive reaction in distilled water and three positive reactions in petrolatum.
- Cobalt chloride <0.0156% (<156 ppm) patch tests induced 14 positive reactions in distilled water and 14 positive reactions in petrolatum [7].

54.3.1.5 Chromium

In the trivalent state (Cr^{3+}), chromium is an essential trace element, important for several biological processes in humans. Poorly soluble, Cr^{3+} salts are also poor penetrants through biological membranes, including undamaged human skin. In the hexavalent state (Cr^{6+}), mostly of anthropogenic origin, chromium is highly immunogenic, genotoxic, and carcinogenic in mammals. Its salts easily penetrate the skin, particularly in the work environment, thereby causing irritation and allergy of the delayed type.

In a study of 47 chromium-allergic patients (including chromium-sensitive only, chromium- and cobalt-sensitive, chromium-, cobalt-, and nickel-sensitive), the mean threshold concentration of chromium has been evaluated in three different vehicles: distilled water, petrolatum, and alkaline aq. buffer (pH 12).

- Potassium chromate 0.0312% (312 ppm) in distilled water: patch tests were positive in one patient, in buffer in eight patients, and in petrolatum in seven patients.
- Potassium chromate 0.0156% (156 ppm) in distilled water: patch tests were positive in seven patients, in buffer in five patients, and in petrolatum in three patients.
- Potassium chromate 0.0078% (78 ppm) in distilled water: patch tests were positive in two patients, in buffer in nine patients, and in petrolatum in two patients.
- Potassium chromate 0.0039% (39 ppm): patch tests were all negative, except for in one patient in buffer.
- Potassium chromate <0.0039% (<39 ppm) in distilled water: patch tests were positive in two patients, in buffer in eight patients, and in petrolatum in two patients [7] (Table 54.1).

TABLE 54.1
Metals

Materials	Screening Concentration	Results (Positive/ Tested Subjects)	Reference
Mercuric chloride	500 ppm	29/207	5
	500 ppm	27/270	5
	500 ppm	5/5	6
	500 ppm	21/35	6
	500 ppm	5/12	11
	500 ppm	2/2	10
	500 ppm aq.	2/13	7
	250 ppm aq.	3/13	7
	250 ppm pet.	4/13	7
	31 ppm pet.	2/13	7
	15 ppm aq.	3/13	7
	15 ppm pet.	2/13	7
Phenyl mercuric acetate	500 ppm	101/1852	16
	500 ppm	(5.5%)	12
	100 ppm	8/19	16
	100 ppm	192/10974	15
	100 ppm	(1.7%)	15
		54/1349	
		(4%)	
		388/10486	
		(3.7%)	
Thiomerosal	500 ppm	778/19454	16
	500 ppm	(4.0%)	16
		440/9361	
		(4.7%)	
Thimerosal	500 ppm	12/1025	13
	100 ppm	3/4	14
Sodium timerfonate	500 ppm	31/6548 (0.5%)	16
Ethylmercury chloride	500 ppm	138/1025	13
	310 ppm	18/18	17
	165 ppm	19/21	19
	165 pm	32/32	18
	150 ppm	18/18	17
Methylmercury chloride	310 ppm	19/21	19
	310 ppm	36/36	17
Phenylmercury nitrate	500 ppm	3/19	12
Gold sodium thiosulfate	500 ppm	8/373 (2.1%)	23
	500 ppm	38/1203	22
	500 pm	(3.2%)	22
	50 ppm	5/105	4
		16/23	
Nickel chloride	200 ppm	4/17	28
	100 ppm	4/17	28
Nickel sulfate	390 ppm	4/35	31
	<390 ppm aq.	5/53	29
	190 ppm	6/35	31
	112 ppm	9/25	25
	100 ppm	19/329	27
	50 ppm	5/92	27
	32 ppm	4/15	32
	10 ppm	4/92	27
	10 ppm	12/20	25
	5 ppm	6/12	25
	1 ppm	3/20	25
	0.5 ppm	2/20	25
Cobalt chloride	312 ppm aq.	8/60	7
	312 ppm pet.	8/60	7
	156 ppm pet.	3/60	7
	<156 ppm aq.	14/60	7
Potassium chromate	312 ppm buff.	8/47	7
	312 ppm pet.	7/47	7
	156 ppm aq.	7/47	7
	156 ppm buff.	5/47	7
	156 ppm pet.	3/47	7
	78 ppm aq.	2/47	7
	78 ppm buff.	9/47	7
	78 ppm pet.	2/47	7
	<39 ppm aq.	2/47	7
	<39 ppm buff.	8/47	7
	<39 ppm pet.	2/47	7

Source: Reprinted with modifications from Jerschow, E., Hostynek, J.J., and Maibach H.I., *Food Chem. Toxicol*., 39(11), 1095–1108. With permission.
Note: aq.—aqueous solution, pet.—petrolatum, buff.—buffer.

54.3.2 Botanicals

Primin has been included in the European standard series of patch tests since 1985, as contact dermatitis from the plant *Primula obconica* became common in western Europe and Scandinavia. Increasing the test concentration beyond 0.01% (100 ppm) was found unadvisable as reactions to primin 0.01% (100 ppm) are already strong, and raising the concentration further would increase the risk of active sensitization by patch testing. Primin 0.01% (100 ppm) patch tests were positive in 57 of 3075 random dermatological patients [33].

Contact dermatitis due to plants of the Araliaceae family has been reported, and the major allergen in these plants was shown to be falcarinol. At 0.03% (300 ppm) falcarinol in petrolatum, patch tests elicited 4/4 positives in patients allergic to the Araliaceae family [34].

Cross-allergy among these plant constituents has been determined in a study of sensitized Japanese farmers and of previously healthy control subjects who had been sensitized

TABLE 54.2
Botanicals

Materials	Screening Concentration (ppm)	Results (Positive/Tested Subjects)	Reference
Primin	100	57/3075	33
Falcarinol	300	4/4	34
cis-9,17-Octadecadiene-12,	500	6/7	36
14-diyne-1,16-diol	500	4/5	36
	100	5/5	35
16-Hydroxy-*cis*-9,17-	100	4/5	35
octadecadiene-12,14-diynoic acid	100	2/5	35
Tulipalin A	300	3/3	37
Urushiol	100	6/8	38
	100	4/7	36
Dehydrocostus lactone	330	4/10	39

Source: Reprinted with modifications from Jerschow, E., Hostynek, J.J., and Maibach H.I., *Food Chem. Toxicol*., 39(11), 1095–1108. With permission.

by patch testing. Compounds from the evergreen plants *Dendropanax trifidus* and *Fatsia japonica*, belonging to the family Araliaceae, led to the following patch tests results (read on days 2, 3, and 7) [35].

- 0.01% (100 ppm) *cis*-9,17-octadecadiene-12,14-diyne-1,16-diol—5/5 positive
- 0.01% (100 ppm) 16-hydroxy-*cis*-9,17-octadecadiene-12,14-diynoic acid—4/5 positive
- 0.01% (100 ppm) *cis*-9, *trans*-16-octadecadiene-12,14-diynoic acid—2/5 positive

0.05% (500 ppm) *cis*-9,17-octadecadiene-12,14-diyne-1,16-diol elicited six of seven positive reactions in patients allergic to the plants of Araliaceae family, and also in four of five control subjects [36].

Tulipalin A (α-methylene-γ-butyrolactone) was identified as the allergen in tulips and alstromeria. Patch testing at the level of 0.03% (300 ppm) in ethanol elicited three positive reactions out of three horticulturists presenting with skin lesions [37].

Another well-known plant allergen from urushi plants is urushiol. Patch testing with urushiol at 0.01% (100 ppm) in petrolatum (exposure time 2 days, reading on day 3) gave positive reactions in six of eight forest workers also allergic to 2,2′-azobis(2-aminopropane) dihydrochloride [38]. There is a suggestion about cross-reactivity to urushiol in patients with contact dermatitis due to *Dendropanax trifidus* (Araliaceae family): four of seven patients showed strong positive reactions when patch tested with 0.01% (100 ppm) urushiol [36].

Dehydrocostus lactone is a component of *Compositae* mix that is often responsible for *Compositae* dermatitis in gardeners. Dehydrocostus lactone at 0.033% (330 ppm) petrolatum patch tests were positive in 4 of 10 Danish florists with occupational dermatitis [39] (Table 54.2).

54.3.3 Biocides

As a group, chemicals that have biocidal (antimicrobial) properties and are used as disinfectants, preservatives, pesticides and are otherwise useful in deterring, reducing, or eliminating undesirable or dangerous organisms, have one characteristic in common: they are highly protein-reactive, and their activity is based on changing or destroying basic functions of target organisms. Since the demise of the effective and popular antibacterial hexachlorophene in the early 1970s, mainly the isothiazolinones 5-chloro-2-methyl-4-isothiazolinone (MCI), 2-methyl-4-isothiazolinone (MI), 1,2-benzisothiazolinone (BIT), and methyltrimethylene isothiazolinone (MTI), have taken its place. Applications for isothiazolinones as stabilizers of aqueous media have proliferated: preservatives in household and industrial products such as metal-working fluids, cooling-tower water, latex emulsions, paper mills, and most significantly for consumers, in cosmetics and toiletries. Commensurating with their rate of application, the literature reports on their allergenic action have also increased.

From 1983 to 1986, 365 patients with suspected sensitization to MCI/MI were patch tested with the biocide. Twenty patients had positive reactions at 100 ppm MCI/MI aq. and in petrolatum [40].

MCI/MI: 0.005% (50 ppm) patch tests were positive in 10 of 24 patients; at 0.0025% (25 ppm) patch tests were positive in 9 of 24 patients [41].

MCI/MI: 0.01% (100 ppm) patch tests elicited 24 of 24 positive reactions in patients believed to be allergic to the antimicrobial [41].

MCI/MI at 0.0015% (15 ppm) in the provocative use tests (PUT) in rinse-off products (six applications daily for 14 days): positive in 3 of 27 persons tested.

MCI/MI at 0.0025% (25 ppm) in the PUT in rinse-off products: positive in four of four persons tested.

MCI/MI 0.0015% (15 ppm) PUT in leave-on products (two applications daily for 7 days): positive in 31 of 107 tested.

MCI/MI at 0.0007% (7 ppm) in the PUT in leave-on products (two applications daily for 14 days): positive in 52 of 52 tested.

MCI/MI at 0.015% (150 ppm) in the PUT in leave-on products (two applications daily for 14 days): positive in 4 of 567 persons tested [42].

Patch testing of seven individuals hypersensitive to MCI/MI with serial dilutions of the biocide gave following results:

- 200 ppm of MCI/MI—seven of seven patients had positive reactions
- 100 ppm—five of seven patients reacted positively
- 50 ppm—four of seven, and 25 ppm—two of seven patients had positive reactions [43]

MCI/MI at 0.01% (100 ppm) patch tests, read on days 2, 3, and 4–7: positive in 15/590 patients with ACD; 0.02% (200 ppm) patch tests—positive in 16/589 patients [42].

MCI/MI as 0.01% (100 ppm) patch: tests were positive in 3% of 3078 patients suspected with ACD [44].

MCI/MI as 0.01% (100 ppm) patch: tests were positive in 2.5% of 2110 women with medical occupations [15].

Sixteen of 225 MCI/MI-sensitized subjects reacted to 100 ppm [45].

MCI (chloromethylisothiazolinone) 0.002% (200 ppm) patch tests gave positive reactions in two of 45 sensitized subjects. MTI at 0.03% (300 ppm), patch tests were positive in three of 19 [46]. In random dermatological population tested to 0.03% (300 ppm) octylisothiazolinone, 7 of 1556 patients had positive reactions [47].

Of 1094, 4.2% children tested positive to patch testing with 100 ppm of MCI [48].

BIT as 0.04% (400 ppm) aq. patch, exposure time—2 days, reading on days 3 and 4: positive in 4 of 17 occupational contact dermatitis patients; 10 of 537 patch tests were positive [49].

BIT as 30 ppm patch: 10 of 556 patch tests were positive in random dermatological population [50].

BIT as 0.05% (500 ppm) alcohol solution has been tested among employees at a manufacture of air fresheners (exposure time 2 days, reading on day 3): three of four workers showed positive reactions. In the same group, three of five workers reacted positively to 0.03% (300 ppm) Proxel CRL (ethylenediamine (24%) and BIT (23%) solution) [51].

In a trial, the eliciting threshold concentration of formaldehyde in formaldehyde-sensitive individuals was studied by the occluded and nonoccluded patch test in serial dilution; also the relationship to repeated open application test (ROAT) with a product containing a formaldehyde releaser was evaluated. Formaldehyde at 0.025% (250 ppm) occluded patch test (exposure 2 days, reading on days 2, 3, 6–9) gave positive readings in three of four. No positive reactions were observed in the non-occluded patch test or the ROAT [52] (Table 54.3).

54.3.4 Corticosteroids

The allergenic response to low concentration of corticosteroids may be muted due to their anti-inflammatory properties, which can protect skin from inflammatory signs. Low concentrations of corticosteroids may nevertheless produce strong positive reactions [53] since an anti-inflammatory level may not have been reached. Budesonide 0.0002% (2 ppm) patch tests (occlusion time 48 h, reading time 2 and 4 days): 8/10 (80%) positive; 0.002% (20 ppm) patch tests—8/10 (80%) positive; 0.02% (200 ppm) patch tests—7/10 (70%) positive [51]. For comparison, budesonide 2% (20,000 ppm) patch test with the same occlusion and reading time only elicited five positive reactions in 10 subjects tested (50%). In a different study, where patch tests also remained under occlusion for 48 h and reading has been done on day 3, the results were as follows: budesonide 0.001% (10 ppm)—19 of 26 tested subjects reacted positive (73%); budesonide 0.01% (100 ppm)—20 of 26 subjects had positive reactions (78%) [54] (Table 54.4).

54.4 DISCUSSION

The database of chemical structures known to cause ACD in humans, which are classified by potency is limited when compared with the more than thousand compounds known to be allergens in animals [55–57]. Of those, allergens known to be of extreme potency number only a few dozen. This may be due to the fact that dermatologists routinely test patients using standard patch test series at a single concentration, which leads to an undifferentiated "yes"/"no" diagnosis for hypersensitivity. Only exceptionally do research dermatologists resort to statistically significant cohorts of human volunteers to establish threshold elicitation concentrations by dose–response design, or by multiple-dose response studies [25,29,58,59]. It may also be, however, that allergens that meet our criterion of "extreme" are uncommon.

Furthermore, clear and unambiguous classification as to allergenic potency of a chemical *per se* is problematic, since it depends on a number of endogenous and exogenous factors:

- Clinical experience suggests the existence of different degrees of sensitivity among patients allergic to the same chemical. Several authors proposed a quantitative concept of "strong" versus "normal" or "weak" contact allergy [60]. According to this concept the skin reactions to different concentrations of a chemical correlate with the grade of the previous sensitization, rendering an individual more (or less) likely to respond to skin contact with a given quantity of an allergen [61,62].
- Intra-individual variation in test reactivity [30].
- Presence (or absence) of active atopic dermatitis in a given individual [63].
- Degree of sweating [64–66].
- Age and gender [67–71].

TABLE 54.3
Biocides

Materials	Screening Concentration (ppm)	Results (Positive/Tested Subjects)	Reference
MCI/MI	200	16/589	42
	200	7/7	43
	150	4/567	43
	100	24/24	42
	100	15/590	41
	100	20/365	42
	100	92/3078 (3%)	40
	100	53/2110 (2.5%)	44
	100	5/7	45
	100	16/225	43
	100	46/1094 (4.2%)	49
	50	10/24	43
	50	4/7	46
	25	9/24	41
	25	4/4	43
	25	2/7	43
	15	3/27	41
	15	33/107 (31%)	42
	7	52/52	42
Chloromethylisothiazolinone	200	2/45	47
Methyltrimethylene isothiazolinone	300	3/19	47
Octylisothiazolinone	300	7/1556 (0.4%)	48
1,2-Benziosothiazolin-3-one	500	3/4	52
	400	4/17	50
	400	10/537	50
	30	10/556	51
Proxel CRL (ethylene-diamine [24%] and 1,2-benzisothiazolin-3-one [23%] solution])	300	3/5	52
Formaldehyde	250	3/4	53

Source: Reprinted with modifications from Jerschow, E., Hostynek, J.J., and Maibach H.I., *Food Chem. Toxicol*., 39(11), 1095–1108. With permission.

Note: MCI/MI—5-Chloro-2-Methyl-4-Isothiazolinone/2-Methyl-4-Isothiazolinone.

TABLE 54.4
Corticosteroids

Materials	Screening Concentration (ppm)	Results (Positive/Tested Subjects)	Reference
Budesonide	200	7/10	54
	100	20/26	55
	20	8/10	55
	10	19/26	55
	2	8/10	55

Source: Reprinted with modifications from Jerschow, E., Hostynek, J.J., and Maibach H.I., *Food Chem. Toxicol*., 39(11), 1095–1108. With permission.

- In addition to gender variability, there is the assumption that the patient's immunological status might be influenced by and vary according to the stage in the menstrual cycle. Studies and case reports have shown increased test reactivity both to allergic and irritant reactions premenstrual [32].
- Anatomic site tested [32,72–74].
- Vehicle [75–77].
- Different patch brands [78].
- Varying amounts of an allergen placed on the skin [79].
- Differences in testing; patch test, provocative use test (PUT), repeated open application test (ROAT) [80].

We note other limitations of data correlation: additional variables include study population (routine screening of dermatitis patients versus aimed testing), literature collection (hand screening), and underreporting.

Finally, there is a suggestion that the threshold for elicitation is not a constant property of an allergen. Remarkably, this effect is seen across species in guinea pigs, mice, and humans in a number of different allergens [81]. This is why it can be difficult to determine clear threshold doses that will or will not elicit allergic responses.

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55 Allergic Contact Dermatitis to Topical Anesthetics: A Cross-Sensitization Phenomenon

Christopher J. Dannaker, Erik Austin, and Howard I. Maibach

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55.1 INTRODUCTION

Since their development in the latter half of the nineteenth century (Dripps et al., 1992), topical anesthetics have been found to be common producers of allergic contact dermatitis (ACD). An incidence of allergic reactions to topical medicaments of up to 15–20%, excluding patients with leg ulcers or other high-risk patients, seems a realistic figure to be expected in a contact dermatitis clinic (Brandao et al., 2001).

Exposure to topical anesthetics may present an occupational hazard to certain workers including ophthalmologists and ophthalmologic technicians.

This chapter discusses ACD to topical analgesics and presents two cases of ACD to proparacaine with subsequent cross-sensitization to tetracaine from ophthalmic preparations. This interesting cross-sensitization phenomenon may be an underrecognized problem of ophthalmologists, related personnel, and patients who are repeatedly exposed to topical ophthalmic anesthetics.

55.2 TOPICAL ANESTHETICS

Topical anesthetics may be classed into esters and amides. The esters that may induce ACD include *p*-aminobenzoic acid, benzocaine, procaine, and tetracaine (Cronin, 1980). There is a cross-reaction of these anesthetics with components of the paragroup, specifically, *p*-phenylenediamine. The amides are considered to be less potent sensitizers; most commonly, lidocaine, bupivacaine, mepivacaine, and prilocaine. Cross-reactions have been reported between lidocaine and mepivacaine, as well as bupivacaine and prilocaine (Weightman and Turner, 1998). Contact sensitization to prilocaine has been primarily induced by EMLA cream (Van der Hove et al., 1994; Le Coz et al., 1996).

55.3 OPHTHALMIC PREPARATIONS

Contact sensitization to ophthalmic preparations is common. Preservatives such as thimerosal were the main cause in eyedrop users and contact lens wearers (Tosti and Tosti, 1988). The active ingredients of the ophthalmic preparations may also cause sensitization, for example, β-adrenergic blocking agents, mydriatics, antibiotics, antiviral drugs, antihistamines, anti-inflammatory drugs, corticosteroids, and anesthetics (Brandao et al., 2001).

ACD to the topical anesthetic proparacaine by ophthalmic mucous membrane exposure has been reported (Herbst and Maibach, 1991, 1992; Brandmann et al., 1974; Brancaccio et al., 1993). However, there have been few cases described in which sensitization to proparacaine occurred by the cutaneous route (March and Greenwood, 1968; Lorenzetti, 1969; Liesegang and Perniciaro, 1999; Cronin, 1980). The first case of ACD to proparacaine was reported by March and Greenwood (1968), occurring in an ophthalmologist and manifesting as finger pad fissuring and eczematous dermatitis of the fingertips. Lorenzetti (1969) described similar clinical findings in a research scientist administering proparacaine during animal studies. Later, a patient with

refractory glaucoma was found to have an iatrogenically induced contact allergy to proparacaine when used as an anesthetic before measuring his intraocular pressure (Liesegang and Perniciaro, 1999). Avoidance of this anesthetic resulted in resolution of the patient's recurrent bouts of periocular dermatitis and conjunctivitis. A practicing ophthalmologist was found to have an allergic response to proparacaine after an extended 3 years search to find the offending agent (Cronin, 1980). Cross-sensitization between proparacaine and tetracaine is thought to be rare. Specifically, proparacaine is suggested to be chemically distinct enough from tetracaine to explain a lack of cross-sensitization (Hardman and Limbird, 1980). Yet, as discussed in Section 55.5, our clinical case presentations (Dannaker et al., 2001) support the concept that cross-sensitization or cosensitization is a distinct possibility.

55.4 LOCAL ANESTHETICS

Local anesthetics can be divided into four major groups according to their chemical structure: benzoic acid ester (e.g., proparacaine; tetracaine), quinoline (e.g., dibucaine), amide (e.g., lidocaine), and other compounds (e.g., propipocaine). Structural formulas for proparacaine, tetracaine, procaine, and benoxinate are shown in Figure 55.1. Proparacaine is an ester of *m*-aminobenzoic acid and tetracaine is of the *p*-aminobenzoic acid class. Local anesthetics of the *p*-aminobenzoic acid derivatives are thought to have strong sensitizing potential (Cronin, 1980).

Benoxinate is a benzoic acid ester related to procaine. Benzocaine resembles procaine, except that it lacks a terminal diethylamine group. Most anesthetics have a hydrophilic group and a hydrophobic aromatic residue separated by an alkyl chain. The linkage to the aromatic group (e.g., ester, amide) determines many of the anesthetic pharmacologic properties. Benzoic acid ester anesthetics have an ester link to the aromatic group. The ester bond is hydrolyzed during metabolic degradation. Procaine is typical in that it can be divided into three parts: aromatic (*p*-aminobenzoic), alcohol (ethanol), and tertiary amino group (diethyl amino) (Hardman and Limbird, 1980).

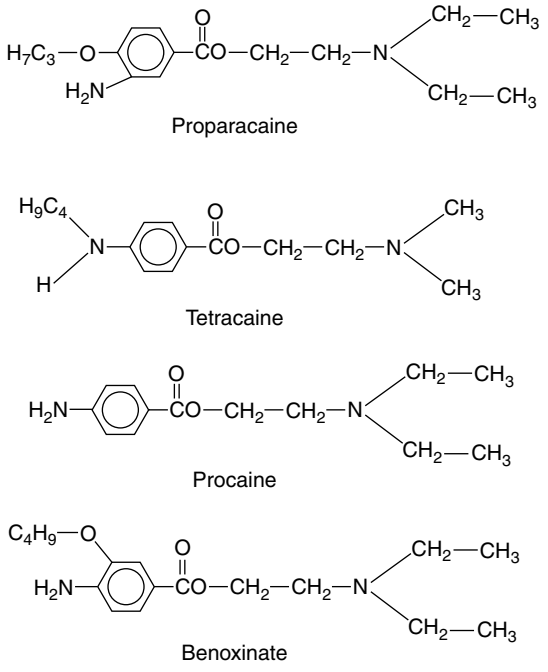


FIGURE 55.1 Chemical structures of anesthetic agents demonstrating similarity of proparacaine with tetracaine and benoxinate with procaine.

55.5 CASE PRESENTATIONS

55.5.1 Case 1

A 58-year-old white male ophthalmologist was presented to the Occupational and Environmental Dermatology Clinic of the University of California at San Francisco, complaining of bilateral fissuring and scaling of the finger pads for 3 years. His thumbs were most affected. Use of topical corticoids failed to ameliorate his symptoms.

Patch testing to the North American Contact Dermatitis Research Group allergens was performed. In addition, the Hermal (Trolab Hermal, Omniderm Inc., Quebec, Canada) local anesthetic tray was tested (Table 55.1). Ophthalmic preparations were tested as is. Routine technology with Finn chambers (Epitest, Helsinki) on Scanpor tape (Norgeplaster, Oslo) was used. Results were read at 48 and 96 h (Table 55.1).

A 3+ spreading reaction occurred at 48 and 96 h to Ophthetic solution 0.5% (proparacaine HCl), but not to the local anesthetic tray or benzocaine. Benzalkonium chloride was not tested separately. Ophthetic solution contains 0.5% proparacaine in a pH-balanced solution containing glycerin, which is preserved with 0.01% benzalkonium chloride. A 1+ reaction

TABLE 55.1
Results of the First and the Repeat Patch Tests (2 Years Later) on an Ophthalmologist

	First Patch Test	Second Patch Test (2 Years Later)
Ophthetic solution 0.5% (proparacaine HCl)	3+/3+	1+/1+
Tetracaine HCl 1% Hermal	Negative	Not tested
Tetracaine HCl 1% eye drops	Not tested	2+/2+
Benzocaine	Negative	Negative
Cinnamic aldehyde and cinnamic alcohol	1+/1+	2+/2+
Fluress (Benoxinate)	Not tested	Negative
Carba mix (3% petrolatum)	Positive	Positive
Thiuram (1% petrolatum)	Negative	1+/1+
Caine mix	Not tested	2+/2+

to cinnamic aldehyde and alcohol (1% petrolatum) also was noted at 48 and 96 h.

A diagnosis of ACD to proparacaine was made, and the patient was instructed to substitute tetracaine, to which he had a negative patch-test result. He was instructed to avoid cinnamon-fragranced/flavored products to minimize cinnamic exposure.

The patient continued to work, but later began to again suffer from sporadic bouts of painful fissuring of his fingertips despite avoidance of proparacaine-containing ophthalmologic medicaments. He also avoided fragranced products and flavored dentifrices (cinnamic aldehyde and alcohol). Nearly complete resolution of dermatitis occurred during a 1-month vacation.

The patient was reevaluated 2 years later. Physical examination revealed fissuring of the finger pads bilaterally. Repeat patch testing was performed.

Results were read at 48 and 96 h (Table 55.1). Results for ophthetic solutions 0.5% (proparacaine HCl) were again positive (i.e., 1+ at both 48 and 96 h). Tetracaine HCl 1% generic brand eye drops caused a reaction of 2+ at both readings. Benzocaine, a *p*-aminobenzoic acid-derived ester anesthetic, again elicited a negative reaction. Other nonanesthetic ophthalmic preparations that the patient used, and were preserved with benzalkonium chloride, elicited negative patch-test reactions. Fluress (Alcorn Inc., Buffalo Grove, Illinois), an ophthalmic solution containing both fluorescein and the topical benzoic acid ester, benoxinate, elicited a negative patch-test reaction. Benoxinate is chemically related to procaine. Cinnamic aldehyde and alcohol again had a positive result. Reactions to carba mix (3% petrolatum) and thiuram (1% petrolatum) were both 1+ at 48 and 96 h. Caine mix tested 2+ at 48 and 96 h.

The patient continued to work as an ophthalmologist, using tetracaine without glove protection. A 2-year follow-up telephone interview revealed complaints of persistent mild finger pad eczematization. An accidental encounter with proparacaine reportedly brought relapse, resulting in severe fissuring.

55.5.2 Case 2

A 60-year-old Asian woman presented to the Occupational and Environmental Dermatology Clinic at the University of California at San Francisco complaining of an allergic reaction to ophthalmic anesthetic drops instilled before laser eye surgery. On examination, there was an eczematous dermatitis of the upper and lower eyelids. Patch testing to the North American Contact Dermatitis Research Group allergens and a topical anesthetic tray were performed.

A 1+ reaction occurred to benzocaine 5% in petrolatum. She denied known exposures to topical benzocaine-containing products. Ophthalmic anesthetic agents to which she had been exposed were applied under standard Finn chambers. Both Alcaine (proparacaine) (Alcon Labs Inc., Fort Worth, Texas) and tetracaine 0.5% were 2+ at 48 and 96 h. These products are preserved with benzalkonium chloride. The patient had a negative patch-test reaction to this preservative at a 0.01% in water concentration when tested separately. The topical anesthetic tray also was tested. The results were positive to the standard 1% tetracaine HCl.

The patient provided no history of exposure to ophthalmic anesthetic agents before her laser eye surgery, but it remains likely she was exposed prior to ophthalmologic examinations. The observation of concomitant ACD to proparacaine and tetracaine again suggests the possibility of cross-reaction or coreaction.

55.6 CROSS-SENSITIZATION PHENOMENON

Given the paucity of previous reports of ACD to proparacaine, the chemical relationship and epidemiology of cross-reactions between other benzoic acid ester topical anesthetics such as tetracaine remain to be clarified. Cases 1 and 2, presented herein, suggest that cross-reaction or coreaction between proparacaine and tetracaine is possible and might be more frequent than previously thought. Our cases of ACD to proparacaine occurred both in an ophthalmologist presenting with fingertip eczematization and in a patient with periocular eczema. Our patient's finger pad fissuring was similar to that reported by March and Greenwood (1968) and Lorenzetti (1969). The clinical presentation of fingertip fissuring is generally felt to represent an endogenous process and was treated as such in our case before patch testing. When ophthalmologists or ophthalmologic technicians are occupationally exposed to topical anesthetic agents, the pattern of dermatitis might be a function of the nature of exposure. When drops are placed in a patient's eye by the physician, one hand is used to suspend the dropper bottle and the other to open the eyelid. Overflow of the liquid anesthetic drops will make contact with the pad of the thumb and, to a lesser extent, other finger pads. The hands might be switched at times, resulting in bilateral involvement. Repeated and concentrated exposure to the allergen with the thumb pad might explain the presence of fissuring.

Despite discontinuance of proparacaine topical anesthetic, the ophthalmologist experienced intermittent hand eczema. Repeat patch testing revealed development of a probable cross-reaction to a related topical anesthetic, tetracaine.

Ophthalmologists and ophthalmologic technicians presenting with chronic finger pad fissuring require patch testing. Repeat patch testing might be necessary when the eczema fails to resolve, as cross-sensitization to related chemicals might have occurred. Working with eczematized skin and a compromised external barrier may result in cross-sensitization to the substituted agent or development of additional contact allergies. This also applies to patients with suspect contact allergies to ophthalmic anesthetic agents and periocular eczema.

It is our belief that sensitization and cross-sensitization to ophthalmic anesthetic agents is more common than previously suspected. Misdiagnosis in ophthalmologists and ophthalmologic technicians caused by the clinical appearance of finger pad fissuring, which simulates endogenous hand eczema, might be common.

55.7 CONCLUSION

We believe that ACD to proparacaine may be an underrecognized problem of ophthalmologists, related personnel, and patients who are repeatedly exposed to topical ophthalmic anesthetics. The clinical pattern for the physician might include fissuring and chronic eczematous changes of the finger-pads that might mimic an endogenous process. For the affected patient, eye irritation with extension of an eczematous dermatitis to the periocular area is typical. The cases herein show that cross-sensitization between proparacaine and other topical anesthetics, such as tetracaine, is possible. Ophthalmologists and ophthalmologic technicians presenting with chronic fissuring require patch testing. Penetration by infectious agents is possible through eczematized skin.

Proper evaluation, use of patch testing, and ingredient substitution is a priority. Because cross-sensitization or cosensitization between proparacaine and tetracaine also might occur, the clinician must be alert to this possibility.

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56 Contact Urticaria and Anaphylaxis to Chlorhexidine: Overview

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56.1 INTRODUCTION

Contact urticaria is a wheal and flare reaction of the skin following external contact to an ever-expanding list of simple chemicals or macromolecules [1], which are usually substances encountered frequently in our environment as preservatives or fragrances and flavorings in cosmetics, toiletries, topical medicaments, and foodstuffs [2,3]. Nonimmunological contact urticaria means a reaction without any previous sensitization and is supposed to be the commonest form. Immunological contact urticaria (ICD) is a Type I hypersensitivity reaction in previously sensitized individuals and is mediated by allergen-specific IgE. Symptoms of ICD span from simple localized wheal and flare to generalized urticaria with involvement of internal organs such as the respiratory or gastrointestinal tract and may culminate in a life-threatening anaphylactic shock [1].

Chlorhexidine (1,1-hexamethylenebis [5-(p-chlorophenyl) biguanide]) has been widely used as a medical disinfectant, for cleansing of both skin and mucous membranes and as an additive for wound dressings for more than 50 years. It serves as a preservative in many cosmetic products, personal care products, and drugs. Gluconate, digluconate, acetate, and diacetate salts of chlorhexidine are equally effective against Gram-positive bacteria and fungi and (to a lesser degree) against Gram-negative bacteria [4]. Another reason for its worldwide use is the small range of reported side effects, usually limited to skin irritation with burning and stinging or staining of teeth and fillings. The allergenic potential has long been underestimated [5]. However, reports of immediate-type reactions to chlorhexidine began to appear more frequently since the 1980s [6]. The prevalence of contact urticaria and anaphylaxis due to chlorhexidine remains unknown. Recent case reports, in which chlorhexidine was suspected only late in the clinical evaluation, suggest that chlorhexidine anaphylaxis is still not well recognized [7].

This chapter reviews the literature on contact urticaria and anaphylaxis to chlorhexidine, paying special attention to the methods by which the diagnosis may be established.

56.2 METHODS

The literature was reviewed using PubMed, entering queries for "chlorhexidine anaphylaxis," "chlorhexidine contact urticaria," and "chlorhexidine allergy." Relevant bibliographic citations from each report so discovered were also reviewed, when possible. In addition, standard textbooks of allergy and dermatology were screened for information about chlorhexidine. Thirteen case reports mentioned in an alert paper by the WHO concerning anaphylactic shock reaction to chlorhexidine-coated central venous catheters without citation[8] and four case reports in Japanese and Swiss journals were not studied in detail. Articles are listed in four tables: Table 56.1 contains cases of reactions to chlorhexidine when applied to wounds or prior to surgery. Table 56.2 lists cases of reactions to chlorhexidine when applied to mucous membranes, Table 56.3 lists reactions to chlorhexidine impregnated central venous catheters, and Table 56.4 lists reactions to chlorhexidine applied to intact skin.

56.3 DISCUSSION

In total we reviewed 72 case reports of immediate type reactions to chlorhexidine.

This is an updated version of the original article that has been published in *Exog. Dermatol.*, 1, 186–194, 2002.

TABLE 56.1
Chlorhexidine Application to Skin

Age	Sex	Indication and Site of Chlorhexidine Use	Immediate Reaction	Chlorhexidine Skin Test Results	Other Test Results	Patient History Concerning Chlorhexidine	Reference
9	M	Preoperative application of chlorhexidine solution	Anaphylactic shock	Open application of 0.05 mL of 1% chlorhexidine to 2 × 2 cm on volar skin showed a wheal after 1 h and a prick test with 0.05% chlorhexidine was positive	Prausnitz-Kuestner test and histamine release test positive		[10]
64	M	Preoperative application and wound dressing with chlorhexidine acetate (0.5%), leg	Erythema and shock Blood pressure 60/40	Intradermal test (0.005% chlorhexidine solution) positive Open application test (0.5%) negative			[22]
24	M	Traumatic wound, application of 0.5% chlorhexidine gluconate, elbow	Generalized urticaria, anaphylaxis, loss of consciousness	Prick test to 0.02% chlorhexidine negative	P-K test and RAST postitive		[17]
15	M	0.5% chlorhexidine on a wound on forehead	Facial urticaria, dyspnea	Scratch test, positive (0.02%)			[23]
26	M	Skin disinfection of the penis prior to circumcision using 1% chlorhexidine solution	Generalized flushing, numbness, dyspnea	Intradermal injection test, positive (0.002 mL 0.0002% chlorhexidine)			[23]
20	F	0.05% chlorhexidine (Merfen blue®) on a wound of the foot	Generalized urticaria, wheezing, angioedema, vomiting, nausea	1% open application positive, Prick test with 0.01% chlorhexidine negative	CAST strongly positive		[12]
48	M	0.5% chlorhexidine to face prior to excision of an artheroma	Urticaria on the eyelids, facial flushing, systemic discomfort	Scratch test positive (0.005%), Patch test (0.5%) positive			[23]
66	M	Skin disinfection before internal jugular vein puncture	Anaphylactic shock, ventricular fibrillation		RAST positive, Lymphocyte transformation assay negative		[18]
12	M	Chlorhexidine 0.5% used for preoperative skin cleansing for hip surgery	Hypotension, tachycardia, generalized rash, facial edema	Scratch test to 0.5% chlorhexidine positive, to 1% chlorhexidine strongly positive			[24]
19	M	Burn in the upper arm: wound disinfection with 0.5% chlorhexidine acetate	Generalized rash, periorbital edema, hypotension, mild angioedema.	Scratch test with 0.05% chlorhexidine positive			[25]
20	M	Two episodes of anaphylaxis Disinfection of a wound with 0.05% chlorhexidine One week later application of a cream containing 0.5% chlorhexidine	Life threatening shock with generalized urticaria, loss of consciousness, need for resuscitation	Prick test with 0.005% chlorhexidine positive	Lymphocyte stimulation test (stimulation index 4,9) positive		[13]

42	M	4% chlorhexidine solution on wound on leg	Skin eruptions, severe hypotension	Skin prick test to chlorhexidine positive[a]			[26]
74	M	4% chlorhexidine solution on wound on leg	Skin eruptions, severe hypotension	Skin prick test to chlorhexidine positive[a]			[26]
18	F	Wound disinfection with a polymerized form of chlorhexidine	Severe anaphylaxis	Prick test to polyhexanide positive[a] prick test to chlorhexidine negative[a]			[27]
15	M	Wound disinfection with a polymerized form of chlorhexidine	Severe anaphylaxis	Prick test to polyhexanide positive[a], to chlorhexidine negative[a]			[27]
53	M	Application of Hibitane (containing 2% chlorhexidine) on the skin in a patient with lung adenocarcinoma after lung resection	Severe hypotension, ECG findings compatible with an inferoposterior infarct but serological exclusion of myocardial necrosis	Skin prick test with 2% chlorhexidine digluconate in 70% alcohol strongly positive			[28]
53	M	Application of 0.05% chlorhexidine gluconate solution to the nasal mucosa prior to surgery	Severe anaphylaxis	Prick test with 0.05% chlorhexidine strongly positive		Skin rash after application of dental gel containing 1% chlorhexidine	[29]
16	M	Disinfection of dermabrasions with chlorhexidine solution	Angioedema, anaphylactic shock	Intradermal test reaction positive with 0.005% chlorhexidine in H20			[30]
43	M	Disinfection of a drain insertion side with chlorhexidine digluconate 2%	Dyspnea, shock, S-T segment elevations	Chlorhexidine digluconate 0.5% patch test positive, prick test with 2% chlorhexidine digluconate in 70% ethanol positive	Lymphocyte transformation assay negative		[15]
49	M	0.05% chlorhexidine bath to treat unspecific dermatitis with nodular prurigo and excoriations	Respiratory arrest, immeasurable blood pressure	Prick test with chlorhexidine gluconate 0.5% positive			[31]
61	M	Skin disinfection prior to hernia operation with chlorhexidine-containing fluid	Urticarial eruption on the back, arms, and stomach	Skin prick test with 1% chlorhexidine gluconate positive Patch testing with chlorhexidine gluconate positive			[32]

[a] Concentration not stated in the reference

TABLE 56.2
Chlorhexidine Application to Mucus Membranes

Age	Sex	Indication and Site of Chlorhexidine Use	Immediate Reaction	Chlorhexidine Skin Test Results	Other Test Results	Patient History Concerning Chlorhexidine	Reference
66	M	Preoperative, genital area and intraurethral anaesthetic gel containing 0.05% chlorhexidine	Urticaria from genital area to trunk and lower extremities	Open test with 0.5% chlorhexidine digluconate, positive only when applied by mistake to slightly eczematous skin, scratch chamber test positive	Scratch chamber tests with Hibi-scrub sponge and Citanest gel positive, with Citanest solution negative		[11]
18	F	Gynecological examination, intravaginal application of a 1% chlorhexidine lubricant (hibitane obstetric cream)	Wheezing, generalized urticaria, edema in eyelids and lips	Open application test with 5% chlorhexidine showed wheal after 1 h and prick test with 1% chlorhexidine positive	Open application test with Hibitane obstetric cream positive		[6]
9	M	0.5%chlorhexidine on urethral orifice and palpebra	Generalized urticaria, bronchospasm, shock	Scratch test, positive (0.05%) Patch test (1%) positive			[23]
9	M	0.05% chlorhexidine on a trauma on the lip	Generalized urticaria, cough, general fatigue	Intradermal injection test, positive (0.002%)			[23]
31	F	0.5 chlorhexidine intravaginal	Generalized urticaria, dyspnea, abdominal pain	Scratch test, positive (0.05%)			[23]
61	M	Application of urinary catheter gel containing 0.05% chlorhexidine	Hypotension, generalized rash, and edema	Scratch test with chlorhexidine digluconate 0.05% positive		Report of two anaphylactic reactions during catheterization	[33]
58	M	Opthalmic wash solution	Anaphylactic shock				[34]
52	M	Two episodes of anaphylaxis chlorhexidine containing urinary catheter lubricant	Anaphylactic shock	Intradermal test 0.001% with chlorhexidine and Prick test with 0.05% chlorhexidine positive			[35]
77	M	Pelvic disinfection with 0.05% chlorhexidine and introduction of 0.05% chlorhexidine containing jelly for cystoscopy	Light headedness, itchy macular rash, hypotension	Intradermal test 0.0005% chlorhexidine gluconate positive			[36]
72	M	Pelvic disinfection with 0.05% chlorhexidine and introduction of 0.05% chlorhexidine containing jelly for cystoscopy	Tightness, wheeze, shortness of breath, periorbital edema, rash on his abdomen hypotension	Intradermal test 0.0005% chlorhexidine gluconate positive			[36]
70	M	Pelvic disinfection with 0.05% chlorhexidine and introduction of 0.05% chlorhexidine containing jelly for cystoscopy	Generalized urticaria, hypotension,	Intradermal test 0.0005% chlorhexidine gluconate positive			[36]
69	M	Pelvic disinfection with 0.05% chlorhexidine and introduction of 0.05% chlorhexidine containing jelly for cystoscopy	Dizzyness, pruritus on back and abdomen	Intradermal test 0.0005% chlorhexidine gluconate positive			[36]

69	M	Pelvic disinfection with 0.05% chlorhexidine and introduction of 0.05% chlorhexidine containing jelly during cystoscopy	Hypotension, chest pain,	Intradermal test 0.0005% chlorhexidine gluconate positive			[36]
80	M	Pelvic disinfection with 0.05% chlorhexidine and introduction of 0.05% chlorhexidine containing jelly during cystoscopy	Hypotension, bronchospasm	Skin testing declined		Similar situation 3 years before during cystoscopy	[36]
30	M	Antiseptic dental gel containing 1% chlorhexidine	Anaphylactic symptoms	Prick test positive to 0.5% chlorhexidine gluconate			[37]
64	M	0.05% chlorhexidine intraurethral prior to transurethral resection of the prostate	Macular rash, severe hypotension	Skin prick test to chlorhexidine gluconate (0.0005%)	Detection of anti-chlorhexidine IgE	One year before two episodes of malaise and macular rash during cystoscopic examination	[19]
75	M	Urethral jelly containing 0.05 chlorhexidine	Hypotension, bronchospasm, tachycardia	Tests after third occurrence of anaphylaxy deemed as unethical		Transural resection with rash bronchospasm, cystoscopy with rush, cardiac arrest due to chlorhexidine containing urethral jelly	[38]
76	M	Application of urinary catheter gel containing 0.05% chlorhexidine (Instillagel)	Generalized pruritus, generalized urticaria, bronchospasm, hypotension, unconsciousness	Prick test with 0.5% chlorhexidine digluconate positive; with 0.05% negative; patch test after 20 min negative	Prick test to Instillagel positive; to Instillagel dilution 1:10 negative		[39]
25	M	0.05% chlorhexidine intraurethral prior to catheterization	Generalized pruritic rash, wheeze, dizziness	Prick test to 1% chlorhexidine positive	Detection of anti-chlorhexidine IgE	History of itchy rash after chlorhexidine	[7]
45	F	Instillation of a catheter gel containing 0.05% chlorhexidine digluconate prior to cystoscopy	Generalized urticaria, angioneurotic edema, stridor	Chlorhexidine 0.5% in H20 prick test positive			[40]
50	M	Two episodes of anaphylaxis Cleansing of the genital area with Savlon (containing chlorhexidine) Insertion of a chlorhexidine impregnated central venous catheter	Generalized rash, periorbital edema, hypotension, culminating in cardiac arrest during the second attack	Prick test with 4% and 0.4% chlorhexidine positive, after the second anaphylactic reaction positive with 0.04% chlorhexidine			[41]
87	M	Disinfection of the genital area with 0.5% chlorhexidine and application of urine catheter gel containing 0.05% chlorhexidine	Blood pressure 60/40, generalized rash, tongue swelling	Prick and patch test with 0.5% chlorhexidine positive		Patient had twice experienced generalized urticaria and itching postoperatively	[16]
51	M	Preoperative disinfection of the genital area with chlorhexidine and intraurethral anaesthetic gel containing 0.05% chlorhexidine	Blood pressure 60/40, rash	Prick test with 0.5% chlorhexidine positive			[16]

(*Continued*)

TABLE 56.2 (continued)
Chlorhexidine Application to Mucus Membranes

Age	Sex	Indication and Site of Chlorhexidine Use	Immediate Reaction	Chlorhexidine Skin Test Results	Other Test Results	Patient History Concerning Chlorhexidine	Reference
69	M	Insertion of a central venous line, an arterial line, and a urinary catheter, preceded by skin disinfection with chlorhexidine 0.5%	Hypotension, tachycardia, urticaria on the legs	Prick test with chlorhexidine 0.5% negative, intradermal test positive with 0.0002%. Patch test positive		Two episodes of ventricular fibrillation and generalized urticaria in connection with invasive procedures	[16]
26	F	Use of Hibitane obstetric cream containing chlorhexidine 1% prior to vaginal examination	Anaphylactic shock	Prick test with 0.1% chlorhexidine positive		Local burning during former examination	[42]
37	F	Cleansing with 0.05% chlorhexidine solution prior to insertion of a urinary catheter	Generalized urticaria and dyspnea	Prick test with 0.5% chlorhexidine diacetate and digluconate positive			[43]
70	M	Cleansing of the penile skin with chlorhexidine 1% and use of lubrication gel containing 0.05% chlorhexidine prior to insertion of a urinary catheter	Profound hypotension, severe bronchospasm, shock reaction	Prick test with chlorhexidine strongly positive			[44]
61	M	Insertion of a central venous line and a urinary catheter, hemicolectomy, preceded by skin disinfection with chlorhexidine 0.5%	Blood pressure 60/40, tachycardia (125 bpm), rash, edema of the eyelids	Prick test with chlorhexidine positive, intradermal test positive with 0.0002%			[16]
87	M	Urethral catheterization for urinary retention, preceded by application of urethral gel containing 2% lidocaine and 0.05% chlorhexidinedigluconate	Generalized urticaria, angioedema of face and lips, bronchospasm, dyspnoea, tachypnoea, fall of blood pressure	Prick test with 0.2% chlorhexidine digluconate positive	Basophil activation assay positive		[20]
47	M	Application of urethral gel containing chlorhexidine gluconate 0.05% for urethro-cystoscopy	Generalized urticaria, chest tightness	Prick test positive			[45]
67	M	Application of urethral gel containing chlorhexidine gluconate 0.05% for urethro-cystoscopy	Pruritic rash and feeling faint	Prick test positive			[45]
64	M	Application of urethral gel containing chlorhexidine gluconate 0.05% for urethro-cystoscopy	Generalized rash, cough, fall of blood pressure	Prick test positive			[45]
41	M	Urethral catheterization prior to surgery with application of urethral gel containing chlorhexidine gluconate 0.05%	Anaphylactic reaction with unrecordable blood pressure	Prick test positive		Minor skin reaction at the time of a previous angioplasty	[45]

TABLE 56.3
Chlorhexidine Impregnated Central Venous Catheters

Age	Sex	Indication and Site of Chlorhexidine Use	Immediate Reaction	Chlorhexidine Skin Test Results	Other Test Results	Patient History Concerning Chlorhexidine	Reference
		13 Japanese patients with anaphylactoid-type reactions to central venous catheters impregnated with chlorhexidine	One death				[8]
47	F	Two episodes of anaphylaxis Introduction of an chlorhexidine coated central venous catheter, Second intervention using the same catheter (since first prick test was negative and the second one had not yet been reported to the anesthesiologists) 6 weeks later	Refractory hypotension, generalized urticaria	Skin prick test with chlorhexidine: 1 week after first anaphylaxis negative, 6 weeks after the first anaphylaxis positive[a]			[46]
28	M	Two episodes of anaphylaxis central venous catheter impregnated with chlorhexidine, second anaphylactic event 4 weeks later during the same procedure	Skin erythema and edema in upper body, severe hypotension, tachycardia	Skin prick test with 0.01% chlorhexidine positive Skin prick test with 0.001% chlorhexidine weakly positive			[47]
50	M	Two episodes of anaphylaxis cleansing of the genital area with savlon (containing chlorhexidine), Insertion of a chlorhexidine impregnated central venous catheter	Generalized rash, periorbital edema, hypotension, culminating in cardiac arrest during the second attack	Prick test with 4% and 0.4% chlorhexidine positive, after the second anaphylactic reaction positive with 0.04% chlorhexidine			[41]

[a] Concentration not stated in reference.

TABLE 56.4
Chlorhexidine on "Unbroken" Skin

Age	Sex	Indication and Site of Chlorhexidine Use	Immediate Reaction	Chlorhexidine Skin Test Results	Other Test Results	Patient History Concerning Chlorhexidine	Reference
40	M	Chlorhexidine gluconate solution for hand cleansing	Urticaria in the hands, superimposed on a preexisting hand dermatitis	Skin prick with 0.5% chlorhexidine gluconate positive			[48]
15	F	Cleansing skin with chlorhexidine gluconate	Urticarial rash, syncope	Prick test with 0.05% chlorhexidine gluconate and 0.01% chlorhexidine acetate positive		Eczematous reaction following to antiacne preparation containing chlorhexidine	[37]
33	M	Application of 0.6% chlorhexidine to buttocks after riding lesson	Generalized urticaria, dyspnea, loss of consciousness	Skin prick with chlorhexidine 0.2% positive			[49]

Twenty-one reactions occurred when chlorhexidine was applied to damaged skin surfaces such as wounds, burns and dermabrasions (10 cases), or when the skin barrier was broken after application of the disinfectant by surgery or vein puncture (nine cases).

Thirty-two patients showed an immediate type reaction when chlorhexidine was applied to mucous membranes. Most occurred after intraurethral instillation of urethral jelly prior to catheterization or cystoscopy (26 cases). Three cases were reported when a chlorhexidine containing lubricant was applied intravaginally prior to gynecological examination. In one case, chlorhexidine was applied to a wounded lip and another, anaphylactic shock occurred after application of an ophthalmic wash solution. Additionally there was a report of anaphylactic symptoms after use of a dental gel containing chlorhexidine.

Sixteen reports, all but one of them from Japan, concerned anaphylactic reactions after introduction of a chlorhexidine-coated central venous catheter. The World Health Organization reported 13 cases in Japan, in one of which the patient died from anaphylactic shock. As a consequence, the company withdrew silver sulfadiazine- and chlorhexidine-coated catheters from the market (Arrowguard®: Arrow) [8]. The U.S. Food and Drug Administration has issued an alert concerning hypersensitivity reactions to chlorhexidine-impregnated medical devices[9].

There have been only three cases in which chlorhexidine use on more or less unbroken skin has led to immediate type allergic reactions. In these cases, chlorhexidine was applied to preexisting hand dermatitis, to acne lesions, or to erythematous skin after friction trauma due to horse riding. Therefore, an intact skin barrier cannot be assumed. Open application tests with chlorhexidine to undamaged skin in patients with immediate type hyperreactivity have been reported in four of the reviewed articles [6,10–12]. Two showed a wheal after 1 h. One was strongly positive (application of 1% chlorhexidine solution)[12]. In one case, the positive result might have been probably due to the mistaken application of chlorhexidine to a slightly exanthematous skin site [11]. Together, these observations suggest that slight damage to the epidermal barrier or application to mucous membranes enhances immediate reaction to chlorhexidine[11].

Dividing the immediate type reactions to chlorhexidine into minor (light headedness, rash, urticaria, dyspnea without life-threatening extent) and major (severe hypotension, anaphylactic shock, loss of consciousness, cardiac arrest, need to resuscitation) we found 7 minor and 14 major reactions on application to broken skin and 16 minor and 16 major reactions on application to mucous membranes. These findings indicate that there is no difference in the strength of reactions on mucosa versus broken skin. In the reviewed literature, there was only one case in which the application of a concentration of 0.05% chlorhexidine on wounded skin led to anaphylaxis. This might be due to the fact that most of the skin surface disinfectants contain chlorhexidine concentrations in the range of 0.5–4% and it shows that application of chlorhexidine to wound surfaces may be able to provoke life-threatening shocks even at its lowest bactericidal concentration [13].

Intriguingly, of all reviewed cases only 15% were females. Bechgaard et al. who tested 2061 patients for contact sensitivity to chlorhexidine, found that it was more common in men (3.2%) than in women (1.9%)[14]. Since patients with chlorhexidine-induced contact dermatitis may be more susceptible to systemic anaphylactic reactions by chlorhexidine[15], this could partly explain the different incidences of anaphylactic reactions to chlorhexidine in men and in women.

In 17 of the 55 case reports studied in detail, more than one severe immediate-type reaction occurred, because chlorhexidine had not been identified as the cause of one or more earlier episodes. The diagnosis was confirmed by prick testing (33 cases), using chlorhexidine concentrations ranging from 0.0005 to 4% (Table 56.5), scratch testing (9 cases), using concentrations from 0.005 to 0.5%, or intradermal testing (11 cases) using concentrations from 0.0002 to 0.005% (Table 56.6). In three patients, two of the mentioned

tests were performed. One of these cases reported a negative prick test with 0.5% chlorhexidine but a positive intradermal test with 0.0002% chlorhexidine solution [16]. Unwanted side effects due to the test method did not occur. In four cases, chlorhexidine-specific IgE was detected using RAST [7,17–19]. In one case chlorhexidine allergy was confirmed by basophil activation test [20]. Advantages and disadvantages of the test methods are listed in Table 56.7.

56.4 IMMUNOLOGICAL MECHANISMS OF CHLORHEXIDINE CONTACT URTICARIA

Chlorhexidine-specific IgE antibodies identified in the serum of affected patients [7,17–19] provide compelling evidence for a Type I immune mechanism in chlorhexidine-induced contact urticaria. Reports of associated generalized anaphylaxis serve further to confirm the presence of classic immediate hypersensitivity [21].

According to the generally accepted scheme, low molecular weight compounds, or haptens, such as chlorhexidine, acquire their immunogenicity by binding to carrier proteins. The resulting hapten–carrier complex is processed by antigen presenting cells (APCs), such as Langerhans cells or macrophages. In genetically predisposed individuals, B lymphocytes are activated to produce antigen-specific IgE by a series of events including cell surface interactions between APCs, B lymphocytes, and TH2 lymphocytes, and a maturation process orchestrated by the cytokines IL-4 and IL-13, which are themselves products of TH2 cells.

Circulating antigen-specific IgE is bound to the high-affinity IgE receptor (FcεRI) on the outer membranes of mast cells and basophils. Antigen cross-linking of receptor-bound IgE leads to receptor aggregation and an influx of calcium ions into the cell, followed by the exocytosis of granule

TABLE 56.5
Prick Test Results to Chlorhexidine

Concentration	Number of Tested Patients	Negative Reactions	Number of Control Tests in Healthy Volunteers
0.0005%	1	—	—
0.005%	1	—	—
0.01%	2	1 [12]	—
0.02%	1	1 [17]	—
0.04%	1	—	2 [41]
0.05%	3	—	1 [35]
0.2%	1	—	3 [49]
0.4%	1	—	2 [41]
0.5%	9	1 [16]	≥2 [39]
1%	3	—	—
2%	2	—	—
4%	1	—	2 [41]

TABLE 56.6
Intradermal Injection Test Results to Chlorhexidine

Concentration	Number of Tested Patients	Negative Reactions	Number of Control Tests in Healthy Volunteers
0.0002%	3	—	—
0.0005%	5	—	5 [36]
0.002%	1	—	10 [23]
0.005%	2	—	—
0.05%	1	—	—

TABLE 56.7
Advantages and Disadvantages of the Test Methods

Test Method	Advantages	Disadvantages
Open test on intact skin	Highly likely to be clinically relevant as no history of false positives to date	
Open test on slightly damaged skin[50]		Unfortunately, no controlled studies available; a positive result could be either ICU or NICU. Uncontrolled studies suggest the former more likely than the latter
Prick test	Literature suggests that chlorhexidine 0.0005–0.2% as aqueous solution does not lead to nonspecific positive results (10 negative controls)[35,39,41,49]	
Intradermal injection test	Literature suggests that chlorhexidine 0.0002–0.002% as aqueous solution does not lead to nonspecific positive results (15 negative controls) [23,36]	
Scratch test	No negative controls reported; danger of nonspecific positive results	
Specific IgE (RAST) and basophil activation test		Procedures not commercially available

contents and the release of mediators including histamine, prostaglandins, and leukotrienes, which produce both the wheal and flare of local urticaria, and the widespread systemic physiologic alterations of generalized anaphylaxis.

Chlorhexidine's chemical structure suggests a hypothetical mechanism whereby two FcεRI-bound IgE molecules might attach to opposite sides of the same chlorhexidine dimer. In this scenario, elicitation of contact urticaria would not require linkage between chlorhexidine and a carrier protein. Rapid cross-linking of a large number of Fcε receptors by free chlorhexidine might lead to sudden receptor aggregation and vigorous degranulation.

56.5 SUGGESTIONS FOR FURTHER RESEARCH

If it could be demonstrated, chlorhexidine-induced degranulation of basophils taken from patients with chlorhexidine contact urticaria, in an *in vitro* system without potential carrier proteins, would support the hypothesis that the dimeric chemical structure of chlorhexidine confers the capacity to elicit contact urticaria directly, without the requirement for hapten–carrier linkage. That information would represent a significant extension of our immunopathogenetic understanding of potentially life-threatening hypersensitivity reactions to this low molecular weight chemical, and might eventually serve to guide the development of safer topical disinfectants.

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57 Immunoadjuvants in Prospective Testing for Contact Allergens

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57.1 INTRODUCTION

In this chapter, we deal with immunological adjuvants that are used, or might be used to test the ability of substances to induce allergic contact dermatitis (ACD) in man. Immunological adjuvants are substances that upregulate the induction of the immune response to an allergen (usually, a hapten) or to a complete antigen. In the case of contact allergens, the incidence or intensity of the delayed-type hypersensitivity (DTH) skin reactions are increased, when the sensitization includes immunoadjuvants, as compared to reactions in controls.

Initially, prospective testing of substances for their allergenicity was performed with volunteers in groups of 10–200 individuals (Draize et al., 1944; Schwartz, 1960). Conditions were arranged in one or more ways so that the likelihood of sensitization was increased: (1) the concentration of the test substance at induction was increased; (2) the material was repeatedly applied to the sensitization site; (3) the barrier layer was damaged by scraping, stripping or with detergent, or rendered more permeable by occlusion; (4) the inflammation in the sensitization site was induced by sodium lauryl sulfate (SLS), freezing, or scraping (Kligman, 1966a,b; Marzulli and Maihach, 1983; Kligman and Epstein, 1975). However, classical immunoadjuvants were not used with test subjects.

Challenge was by patch test, often under occlusion, where the test material was usually presented at a high nonirritating concentration, not necessarily representing the use exposure. All of these maneuvers were designed to increase the sensitizing rate so that reasonable extrapolation could be made from small numbers of subjects to very large groups. Appropriate extrapolation from the harsh sensitization conditions of prospective testing to the real world of allergen exposure requires experience and much common sense.

The prospective testing of substances for their allergenicity in experimental animals can be done successfully without immunoadjuvants. The guinea pig nonadjuvant testing methods such as those of Driaze (a modification of Landsteiner's technique) or of Buehler rely on multiple injections or applications of the test material to the sensitization site to enhance the acquisition of DTH to the allergen (Landsteiner and Jacobs, 1935; Driaze et al., 1944; Draize, 1959; Buehler, 1965; Buehler and Griffith, 1975).

These nonadjuvant testing methods are excellent for the identification of strong, moderate, and sometimes of weak sensitizers; however, very weak sensitizers may be missed (Magnusson and Kligman, 1969, 1970; Klecak, 1983; Marzulli and Maguire, 1982). For the purpose of nonspecifically magnifying the allergenicity of test materials so as to render very weak allergens sensitizing in at least some test animals, immunoadjuvants are required. It is our intention to discuss immunoadjuvants that have been or might be used to enhance the acquisition of ACD (and photoallergic contact dermatitis) in laboratory animals for prospective testing.

A list (not exhaustive) of immunoadjuvants that increase the acquisition of ACD reactions in experimental animals are given in Table 57.1 The mechanisms of these immunoadjuvants are not well understood. However, there is currently considerable research aimed at gaining a better understanding of how these and other immunoadjuvants magnify the immune response and considerable progress has been

TABLE 57.1
Immunoadjuvants for Prospective Testing

1. Complete Freund's adjuvant
2. *Propionibacterium acnes* (*C. acnes*)
3. Cyclophosphamide
4. Local cancer chemotherapeutic drugs
5. Recombinant cytokines
6. Antibody to IL-10
7. Plasmids expressing cytokines
8. Ligands for toll-like receptors
9. Neutralizing antibody to CD4+ CD25+ regulatory T cells (TLRs)

achieved in this area in the last decade. None-the-less, much immunoadjuvant research is trial and error, and the justification for the specifics of particular laboratory protocols is largely based on empirical findings.

lmmunoadjuvants can enhance either the B- or the T-cell response or both responses. lmmunoadjuvants such as alum and incomplete Freund's adjuvant (paraffin oil plus emulsifier), which are primarily B-cell stimulants, are not of practical value for upregulating ACD and are not discussed further.

The guinea pig is the classical experimental animal for DTH to contact allergens and for prospective testing of possible sensitizers. The mouse model of Kimber et al., which is gaining currency, in its present form does not use immunoadjuvants (Asherson and Ptak (1969), Chapter 58, this volume). Other possible experimental animals such as the rat and rabbit are not used, for practice laboratory reasons. Viable Bacillus Calmette-Guerin (BCG) injected into the sensitization site has been reported to increase the acquisition of allergic contact to dinitrochlorobenzene in patients with advanced cancer, but for safety reasons is not suitable for prospective testing (Berd et al., 1982). Further, it is unlikely, for ethical and regulatory reasons, that BCG or other injectable immunoadjuvants would be used for prospective testing in man. Our discussion here of immunoadjuvants for prospective testing of contact allergens is necessarily focused on the guinea pig.

There are two underlying, substantially proven, assumptions of prospective testing of materials for their allergenicity in the guinea pig: (1) that the allergenicity scores of test materials in the guinea pig and in man are the same (strong and weak sensitizers of the guinea pig, in parallel, are strong and weak sensitizers of man) and (2) that the rank order of sensitization rates is not changed by the use of immunoadjuvants, i.e., there is roughly equal magnification by immunoadjuvants of the immune response to different contact allergens. There is good evidence for this second assumption in the case of complete Freund's adjuvant (CFA) but not for the other adjuvants listed in Table 57.1.

57.2 COMPLETE FREUND'S ADJUVANT

Incomplete Freund's adjuvant is paraffin oil with the addition, usually, of a water-in-oil emulsifier (e.g., Arlacel A). CFA is incomplete Freund's adjuvant plus heat-killed tubercle bacilli. CFA very strongly and consistently increases DTH responses. Its origin derives from the observation in the guinea pig more than 80 years ago that the injection of viable tubercle bacilli into the skin rendered that skin much more efficient for the induction of DTH to proteins that were later injected into the same sensitization site (Dienes and Schoenheit, 1926; Dienes and Mallory, 1932). Later, it was found that strong DTH to tuberculin and to other proteins could very efficiently be induced utilizing killed tubercle bacilli in paraffin oil; unsaturated oils were unsatisfactory vehicles (Couland, 1935; Saenz, 1939; Freund and McDevitt, 1942). The finding that haptenized (picrylated) spleen cells emulsified in Freund's complete adjuvant induced very strong ACD to picryl chloride (Landsteiner and Chase, 1941; Chase, 1954) extended the applicability of CFA to contact allergens. Further, it was discovered that sensitization to contact allergens could also be intensified by the intradermal injection of Freund's complete adjuvant and the application of the sensitizer over the "injected skin site." This finding allowed complete Freund's adjuvant to be used as an immunopotentiator for essentially all possible contact allergens and photocontact allergens and it is an important element in the construction of the Magnusson–Kligman guinea pig maximization test (Magnusson and Kligrnan, 1970). lmmunopotentiation by separate administration of allergen and adjuvant requires that the sites of CFA and of contact allergen drain into the same regional lymph nodes; for an immunoadjuvant effect, contact with the skin of CFA and allergen is not necessary (Maguire, 1972, 1974).

Currently, CFA is used in a number of prospective tests in the guinea pig (Klecak, Chapter 52 on this volume). An important consideration is that CFA does not perturb the relative ranking of the contact allergens (Magnusson and Kligman, 1970; Marzulli and Maguire, 1982); weak allergens (remain relatively weak vis-à-vis strong allergens, as assessed by the incidence and intensity of the challenge reactions). A major advantage of CFA techniques in various prospective tests that use them is that they are easily learned and performed. Drawbacks of CFA are its induction of chronic cutaneous ulcers at CFA injection sites, and the production of migratory granulomas and of "adjuvant arthritis" (Chase, 1959; Pearson, 1959). These side effects are the major reasons why CFA is disallowed as an immunoadjuvant for prosective testing in a number of countries.

57.3 PROPIONIBACTERIUM ACNES

Killed *Propionibacterium acnes* (*P. acnes*) in a saline vehicle was at one time widely used in the immunotherapy of human tumors and of tumors in experimental animals (Woodruff, 1980). It had an acceptable clinical toxicity profile, but failed to consistently demonstrate significant efficacy in clinical trials and was abandoned. However, in the last decade killed *P. acnes* has reappeared as a therapeutic agent, this time in veterinary medicine as an immunological stimulant (Becker et al., 1989).

In laboratory animals (mice, rats, and guinea pigs), killed *P. acnes* has been shown to regularly heighten the acquisition of DTH to contact allergens, as well as to facilitate the induction of photoallergy (Maguire and Cipriano, 1983; Maguire, 1981; Maguire and Kaidbey, 1982). Chronic cutaneous ulcers at the site of injection do not occur. I have not seen migratory granulomas or "adjuvant arthritis," nor have these, to my knowledge, been reported. In mice, 30 μg of heat-killed *P. acnes* in saline is injected intradermally into the skin and the sensitizing contact allergen is pipetted onto that site. As with CFA, the contact allergen and *P. acnes* do not have to come into contact with one another in the skin, nor do they have to be administered at the same time; however, an absolute requirement is that they share common draining lymph nodes. A substantial advantage of *P. acnes* is its relative lack of toxicity in the doses needed for immunopotentiation. Its potential as an immunoadjuvant for prospective testing in experimental animals of allergens and photo-allergens has not been realized yet. Preparations of killed *P. acnes* (or one or more fractions of the bacterium) could well be a substitute for CFA. Further, in recent studies we have found that heat-killed *P. acnes* is also a very effective immunoadjuvant for the induction of DTH by plasmids that express proteins. In model preclinical and clinical studies, vaccines using recombinant plasmids that express different proteins have been found to be safe and are likely to be effective (Lowrie and Whalen, 2000). A number of plasmids used as expression vectors are currently in clinical trial.

57.4 CYCLOPHOSPHAMIDE

Cyclophosphamide is a substantial immunosuppressant, as are most of the cancer chemotherapeutic drugs. However, it was observed by Maguire and Ettore (1967) and confirmed by Hunziger (1968) that treatment of guinea pigs with cyclophosphamide prior to application of sensitizer resulted in a marked increase in the intensity of the acquired ACD as well as a prolongation of the challenge reactions. Numerous laboratories have confirmed this observation and extended it to other species including chickens, rats, mice, hamsters, and humans, and to DTH to protein antigens (Katz et al., 1974; Maguire et al., 1976, 1979; Maguire, 1980; Jaffee and Maguire, 1981; Berd et al., 1982). The mechanism of immunopotentiation with cyclophosphamide pretreatment seems to rely on the selective inhibition of a population of T cells with specific suppressor activity, now thought to be CD4+CD25+ T cells. (Lutsiak et al., 2005; Ikezawa et al., 2005)

While particular experiments have suggested that cyclophosphamide (Cy) pretreatment would be useful in prospective testing, at least one large-scale study comparing guinea pig tests utilizing CFA with and without Cy pretreatment concluded that Cy pretreatment was not an advantage (Marzulli and Maguire, 1982). Whether Cy would be a benefit in other protocols, such as the lymph node assay of Kimber in mice, remains to be examined. Melphalan is another cancer chemotherapeutic drug with immunosuppressive and immunoadjuvant properties similar to Cy. As taught us by Mocyr and coworkers in a long series of papers, immunologically these two cancer chemotherapeutic drugs have similar immunomodulatory properties (Dray and Mokyr, 1989; Ikezawa et al., 2005).

57.5 LOCAL ANTICANCER DRUGS

Some years ago, Scheper and coworkers made the startling finding that local cancer chemotherapeutic drugs injected into the sensitization site could upregulate the acquisition of contact sensitivity (Boerrigter and Scheper, 1984). The phenomenon, originally described in guinea pigs, also was demonstrated by that group in mice. Not all cancer chemotherapeutic drugs work equally well; some do not work at all (Limpens et al., 1990). The timing of drug in relation to allergen differs from that of immunopotentiation with systemic Cy in that the local drug is typically given after the allergen. The phenomenon can be seen with complete antigens, as well as with contact allergens (haptens). However, it is sensitive to dose of drug: high doses can be inhibitory and low doses can be ineffective. An important issue is whether a general protocol can be worked out, particularly with respect to dose and time of drug that would be applicable to all substances being tested for their allergenicity. An extensive evaluation of local anticancer drugs as immunoadjuvants for prospective testing remains to be done. A large database would be required.

57.6 RECOMBINANT CYTOKINES

Studies of the regulation of the immune response have identified an array of interleukins, as well as of other cytokines (e.g., chemokines) that modulate the activity of T and B cells. The genes for many of the cytokines have been cloned and the recombinant protein products are available for mice and humans. Indeed, future protocols of immunopotentiation for the purpose of prospective testing of substances for their allergenicity and photoallergenicity are likely to include one or more of these recombinant molecules. It is important to remember that nearly all of the cytokines structurally have species specificity. For many, the structural specificity is reflected in specificity of function e.g., human granulocyte macrophage–colony stimulating factor (GM-CSF) and human interleukin (IL)-12 are inactive in the mouse or guinea pig. We shall discuss gamma-interferon (IFN-γ) and IL-12 as model immune potentiators for ACD.

IFN-γ is a homodimeric molecule consisting of two 18-kDa polypeptides. It is variably glycosylated but its biological activity does not require glycosylation. Activated T cells are the predominant source of IFN-γ. IFN-γ has many different activities on cells, all of which appear to be mediated by specific IFN-γ receptors; these receptors are different from the receptors of the α- and β-(type I) interferons. IFN-γ activates macrophages as well as certain other cell types, especially natural killer (NK) cells, and it induces the expression of tumor necrosis factor alpha (TNF-α) as well as certain other cytokines. TNF-α is required for the expression of ACD; well-sensitized mice treated with antibody to

TNF-α at the time of ACD challenge fail to show positive allergic contact reactions (Bromberg et al., 1992).

Playfair et al. (1985) conducted important studies of mouse IFN-γ as an immunological adjuvant in mice, using as antigen the malarial parasite *Plasmodium yoellii* (Playfair and DeSouza, 1987; Heath et al., 1989). They found that IFN-γ was an effective immunoadjuvant when given with antigen, particularly as relates to T-cell responses and the capacity to heighten the activity of a vaccine are based on killed parasites.

Our laboratory studied the immunopotentiation of ACD in the mouse by recombinant murine IFN-γ (Maguire et al., 1987, 1989). IFN-γ injected into the sensitization site heightened the acquisition of DTH to the contact allergens DNFB (1-fluoro-2,4-dinitrobenzene) and oxazolone (4-ethoxymethylene-2-phenyl-oxazolone). In a typical experiment, groups of BALB/c mice were injected intradermally with murine recombinant IFN-γ or with vehicle, and were sensitized by the application of oxazolone to the skin site. They were ear-challenged with a dilute solution of oxazolone 6 days later. Measurements of ear thickness with a micrometer bearing a rachet were made before challenge and 24 h later. The increase in ear thickness over base line in the IFN-γ-primed mice, as against the saline control mice, was highly significant.

This is a standard way to assess DTH to contact allergens in mice and other rodents (Asherson' and Ptak, 1969). Further, we found that a significant immunoadjuvant effect could be realized when IFN-γ was given as late as 2 days after the administration of allergen. This latter finding suggested that immunopotentiation with IFN-γ was not simply the result of the increased expression of major histocompatability surface antigens on antigen-presenting cells; other mechanisms such as stimulation by IFN-γ of the clonalization of antigen-selected T cells in the regional lymph nodes or TH-I polarization of the immune response, could be involved. Further, immunopotentiation of DTH to contact allergens in the mouse with IFN-γ appears to be predominantly a local rather than a systemic event. Thus, if the sites of sensitizing allergen and of IFN-γ are distant (drain to different lymph node basins), immunopotentiation by IFN-γ is lost.

As far as we are aware, studies focused on the immunoadjuvant effect of local IFN-γ have not been done in humans; however, their feasibility (and the possible utility of IFN-γ in vaccines) is suggested by the lack of toxicity of intradermal recombinant IFN-γ when given to patients (Kaplan et al., 1989).

IL-12 is a heterodimeric molecule consisting of 35 and 40 kDa chains. It is secreted by macrophages and dendritic cells, and was initially identified as a factor that stimulated NK cells and, as a separate observatlon cytotoxic cells (Kobayashi et al., 1989; Stern et al., 1990; Trinchieri, 1994). The human and the murine cDNAs have been cloned, sequenced, and expressed. IL-12, like IFN-γ, is relatively species-specific.

We have studied murine recombinant IL-12 as an immunoadjuvant for ACD in the mouse (Maguire, 1995). We have observed that the administration of IL-12 (as opposed to saline) immediately before contact sensitization markedly heightened the induction of ACD as measured by degree of increase in ear thickness at challenge.

1L-12 administ1ation does not upregulate the expression of DTH when given at the time of challenge. As opposed to IFN-γ, we found that administration of 1L-12 at a distant site, as well as locally, enhanced the acquisition of contact allergy to DNFB and to oxazolone. Intraperitoneal IL-12, in mice, is well tolerated in adjuvant doses and is a consistently effective Th-l immunoadjuvant. This gives a considerable advantage to IL-12 over IFN-γ, since immunopotentiation with systemic IFN-γ fails. The field of IL-12 immunobiology is still evolving. Further interesting findings can be expected that will provide the basis for including IL-12 in ACD prospective testing protocols such as that of the local lymph node assay. Other cytokines that are potential candidates for immunopotentiation in prospective testing include IL-l, GM-CSF, and IL-2. In particular contexts, these molecules have been shown to increase the induction of specific T-cell immunity.

57.7 ANTIBODY TO IL-10

A key player in the regulation of the immune response by cytokines is IL-10. It downregulates both immune and nonimmune inflammatory reactions. Thus, the primary irritant reaction to croton oil as well as the DNFB challenge reaction in DNFB-sensitized mice is reduced by IL-10. Further, systemic IL-10 inhibits the acquisition of contact dermatitis to dinitrochloro benzene (DNCB) (Ferguson et al., 1994). Not expectantly, monoclonal antibody that specifically neutralizes IL-l0 upregulates both the acquisition and the expression of ACD (Maguire et al., 1997). Further, IL-10 knockout mice had increased type 1 (Th-1) reactivity to immunogens (Halak et al., 1999).

Systemic anti-10 antibody could be a useful immunoadjuvant for the prospective testing of contact allergens, particularly in mice.

57.8 PLASMIDS EXPRESSING CYTOKINES

In mice, a number of cytokines expressed by plasmids have been shown to upregulate the Th-1 (ACD–DTH) response when given with a plasmid expressing the immunogen. Plasmids with immunoadjuvant activity include plasmids expressing IL-12, GM-CSF, IFN-γ, IL-8, and RANTES (Kim et al., 2000). We have examined a number of plasmids expressing cytokines for their ability to upregulate the induction of DTH to contact allergens in the mouse. The basic design of the experiments was as follows: plasmid was injected intradermally in a clipped site on the back of the mouse, and 1 or 2 days later, the particular sensitizer (generally oxazalone or DNFB) was applied topically to the same site (Kim et al., 2000). By this means, we demonstrated *in vivo* adjuvant activity with plasmids expressing murine recombinant IL-12 (pcIL-12), GM-CSF (pcGM-CSF), and curiously IL-10 (pcIL-10).

The use of plasmids expressing various cytokines and costimulatory molecules is an active area of investigation, particularly as it relates to the design of effective human and veterinary vaccines. (Lowrie and Whalen, 2000). The technology has not yet been applied to the prospective testing of contact allergens.

57.9 LIGANDS FOR TOLL-LIKE RECEPTORS

Toll-like receptors (TLRs) are cell membrane receptors that when stimulated by the appropriate ligand initiate a signaling cascade that results in the cellular secretion of a group of cytokines many of which are proinflammatory and have ACD immuoadjuvant activity. Each TLR responds to a unique group of ligands and, in turn, induces a unique set of cytokines. The TLRs make up part of the innate immune system Pivarsci et al. (2005). So far, there have been 11 functional TLRs described in the mouse and 10 functional TLRs in man.

A number of ligands for particular TLRs are of interest to us for their potential use in prospective testing of materials for their contact allergenicity. Bacterial DNA expressing unmethylated CpG sequences can behave as immunological adjuvants and up regulate the TH-1 immune response associated with DTH (Van Uden and Raz, 2000). The active DNA has been shown to stimulate innate immunity by reaction with a particular TLR9. As a result, there is secretion of a number of proinflammatory cytokines that can upregulate the immune response (Aderem and Ulevitch, 2000; Akira et al., 2001).

Another example of a ligand that stimulates a TLR is imiquimod (Aldara®), a compound utilized in dermatology for the treatment of viral warts, actinic keratoses, and certain select skin cancers. It acts through and stimulates TLR-7 (Hemmi et al., 2002). We observed in both mice and guinea pigs, in a small experiment, that treatment of the sensitization site with Aldera had a significant immunoadjuvant effect. Ligands that activate innate immunity could be used as immunoadjuvants for special cases in prospective ACD testing.

57.10 REGULATORY T CELLS

Regulatory T cells (Tregs) downmodulate the immune response. Most of these cells have the cell surface markings of CD4+CD25+ T cells and are key to preventing (and suppressing) autoimmune disease and allograft rejection (Avani et al., 2005). In depletion and infusion studies of CD4+CD25+ T cells in mice, Dubois et al. (2003) demonstrated that these Tregs were required for the induction of oral tolerance to DNFB (when assayed in terms of ACD). Further, Cavani et al. (2003) studied a group of individuals who were patch-test negative to nickel. Immuno-histochemistry revealed an abundance of CD4+CD25+ cells in their Ni-challenge patch-test sites. CD4+ T cells from the blood of these nonreactors, when stimulated with the appropriate nickel conjugate, gave a vigorous proliferative response, but only when their CD4+ T cells were depleted of CD4+CD25+ T cells.

In mice, we have found that *in vivo* depletion of CD25+ T cells by a rat monoclonal antibody (PC61) markedly heightens the allergic contact sensitivity induced by routine sensitization with DNFB or oxazolone (Maguire et al., 2006). It is likely that inhibition of Tregs in mice or guinea pigs would render them a more responsive animal for ACD prospective testing. This could be useful in particular instances where standard methods give equivocal results.

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58 Local Lymph Node Assay

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58.1 INTRODUCTION

The acquisition of skin sensitization is dependent upon the initiation of an immune response, and specifically a cell-mediated immune response. The relevant events and processes can be summarized briefly as follows. Sensitization is induced when an inherently susceptible individual is exposed topically to an appropriate and sufficient amount of contact allergen. Following entry into the skin, the chemical allergen either directly, or indirectly, associates with protein and is recognized and internalized by cutaneous dendritic cells, the most important of which in this context are epidermal Langerhans cells (LC). It is now clear that LC play several pivotal roles in the generation of cutaneous immune responses and the induction of skin sensitization; their most important responsibility being the transport of antigen, via the afferent lymphatics, to draining lymph nodes. During this migration from the skin, LC are subject to a functional maturation with the result that by the time of their arrival in lymph nodes they have acquired the characteristics of immunostimulatory antigen presenting cells (Cumberbatch et al., 2000, 2003, 2005; Griffiths et al., 2005; Kimber et al., 1998a, 2000). In the lymph nodes, antigen is presented to T lymphocytes and responsive cells become activated and are stimulated to divide and differentiate. Cell division results in a selective clonal expansion of allergen-responsive T lymphocytes; this quantitative increase in specific T lymphocytes represents the cellular basis for sensitization and immunological memory. If the now sensitized subject is exposed again to the same chemical, at the same or a different site, then this expanded population of specific T lymphocytes will recognize and respond to allergen in the skin and trigger an accelerated and more aggressive secondary immune response, that in turn causes the cutaneous inflammation that is recognized clinically as allergic contact dermatitis. The molecular and cellular mechanisms that result in the induction and elicitation of contact allergy have been reviewed extensively elsewhere (Dearman and Kimber, 2003; Grabbe and Schwarz, 1998; Kimber et al., 2002; Kimber and Dearman, 2002, 2003). For the purposes of this chapter it is sufficient to say that the ability of chemical allergens to induce the activation of skin draining lymph nodes and to stimulate lymph node cell (LNC), proliferative responses are the events upon which the local lymph node assay (LLNA) is founded.

There are several review articles that consider various aspects of the LLNA (Basketter et al., 2001a, 2002, 2003, 2005; Basketter and Kimber, 2001; Dearman et al., 1999; Dearman and Kimber, 2004; Gerberick et al., 1999, 2000; Kimber et al., 1994, 2002; Sailstad, 2002). The purposes here are to review the development and subsequent evaluation and validation of the LLNA and to examine the use of this method for hazard identification, potency evaluation, and risk assessment.

58.2 DEVELOPMENT OF THE LLNA

Based upon an appreciation of the events induced during skin sensitization, the initial objective was to determine whether a method for hazard identification could be developed in mice that might be used as a viable alternative to the then favored guinea pig assays. In contrast to those guinea pig methods (in which activity is measured as a function of challenge-induced cutaneous reactions in previously sensitized animals), the strategy adopted was to focus on events during the induction phase of skin sensitization, and in particular on changes provoked in lymph nodes draining the site of exposure. Several parameters of lymph node activation could be viewed as

legitimate potential correlates of skin sensitization, including increases in lymph node weight and cellularity, the appearance of pyroninophilic cells, and the stimulation of LNC turnover (Kimber et al., 1986; Kimber and Weisenberger, 1989). Preliminary investigations revealed, however, that of these the induction of LNC proliferation represented the most sensitive and most selective marker of skin sensitizing activity. In initial studies proliferative activity had been measured *in vitro* during culture of draining LNC with [^{3}H] thymidine (^{3}H-TdR) (Kimber et al, 1986; Kimber and Weisenberger, 1989). However, one important development was to instead measure lymph node hyperplastic responses *in situ* (Kimber, 1989; Kimber et al., 1989). This adaptation not only provided a more holistic and sensitive assessment of cell turnover by LNC, but also served to obviate the need for tissue culture (with consequential logistic advantages). It is this form of the LLNA that has been the subject of extensive evaluations, and that was subsequently validated.

The basic protocol for the LLNA has been described in detail elsewhere (Dearman and Kimber, 2004; Gerberick et al., 1992; Hilton and Kimber, 1995; Kimber, 1998; Kimber and Basketter, 1992), but can be summarized briefly as follows: Mice of CBA strain are used. Groups of mice receive topical applications of various concentrations of the test chemical (or of the relevant vehicle control) daily for 3 consecutive days. Recommendations regarding suitable test concentrations are available elsewhere (Kimber and Basketter, 1992). For the purpose of hazard identification it may be considered desirable to select the highest recommended test concentrations. In practice, however, this is not always possible. Concerns regarding local or systemic toxicity, or poor solubility, may dictate a more conservative approach.

Several vehicles may be used, and again those usually favored are considered elsewhere (Basketter and Kimber, 1996; Basketter et al., 2001b; Dearman et al., 1999; Kimber and Basketter, 1992; Ryan et al., 2002). Decisions regarding the choice of vehicle (in the context of hazard identification at least) are reached usually on the basis of suitability for topical application and the solubility of the test material. It is relevant to mention here that the vehicle in which a chemical allergen is encountered at skin surfaces can have a significant impact on the extent to which skin sensitization is acquired, and on the vigor of responses in the LLNA (Basketter et al., 2001b; Cumberbatch et al., 1993; Dearman et al., 1996; Heylings et al., 1996; Warbrick et al., 1999a; Wright et al., 2001). There is no doubt that the vehicle matrix also influences the elicitation of responses in other methods for the identification of contact allergens. Although vehicle effects have, in practice, little or no impact on the performance of the LLNA in the context of hazard identification, they are (quite rightly) of more significance when considering LLNA dose responses for the purposes of potency and risk assessment. This issue will be addressed again later.

Five days following the initiation of exposure, mice receive an intravenous injection of ^{3}H-TdR. Animals are sacrificed 5 h later and draining auricular lymph nodes excised. These are either pooled for each experimental group, or alternatively are pooled on a per animal basis. Single cell suspensions of LNC are prepared and the cells washed and suspended in trichloroacetic acid (TCA) for at least 12 h at 4ºC. Precipitates are suspended in TCA and transferred to an appropriate scintillation fluid. The incorporation by draining LNC of ^{3}H-TdR is measured by scintillation counting and recorded as mean disintegrations per minute (dpm) for each experimental group, or for each animal. In those instances where it is deemed appropriate to include within the test protocol a positive control, it is recommended that hexyl cinnamic aldehyde (HCA) is used for this purpose (Dearman et al., 1998, 2001).

For each concentration of test material a stimulation index (SI) is calculated using as the comparator the (dpm) value derived from the concurrent vehicle control. Skin sensitizers are defined as those chemicals that at one or more test concentrations, are able to induce an SI of 3 or greater. It must be recognized that the original decision to use an SI value of 3 as the criterion for a positive response in the LLNA was arbitrary; the choice being made on the basis of experience with a range of chemical allergens and nonsensitizing chemicals. However, it would appear that the decision was correct, since continued experience has revealed that in practice an SI of 3 appears to provide an accurate identification of skin sensitizing chemicals. Moreover, a retrospective analysis of results obtained with some 134 chemicals in the LLNA was reported in 1999 (Basketter et al., 1999a). The data were subjected to a rigorous mathematical assessment using receiver operator characteristic (ROC) curves. The conclusion drawn from these analyses was that an SI value of 3 provides an appropriate criterion for the identification of contact allergens (Basketter et al., 1999a). Despite the proven value of an SI of 3 for hazard identification, some flexibility is appropriate when interpreting LLNA data. It has been recommended previously (Kimber and Basketter, 1992) that the characteristics of dose–response relationships and other factors should be taken into account. Thus for instance, if a test chemical were to display a dose-related increase in LNC proliferative activity that just failed at the highest concentration to achieve an SI of 3, then it would in most circumstances be inappropriate to conclude that the material lacked any potential to cause skin sensitization. In such cases it would be prudent to conduct a repeated analysis using, if possible, higher concentrations of the test chemical or a different vehicle.

A summary of the conduct of the standard LLNA is illustrated in Figure 58.1.

Before leaving the conduct of standard assays and considering the performance of the LLNA, it is appropriate to acknowledge that some other investigators have proposed modifications to the basic protocol. Such vary in their scope and complexity. Some suggested changes are relatively modest and conservative, such as the use of an alternative isotope, or nonisotopic methods, for measurement of LNC proliferation (Ladics et al., 1995; Takeyoshi et al., 2001, 2003, 2004, 2005, 2006), or the consideration of the use of mouse strains other than CBA (Woolhiser et al., 2000). However, other proposals call for much more substantial changes to the standard

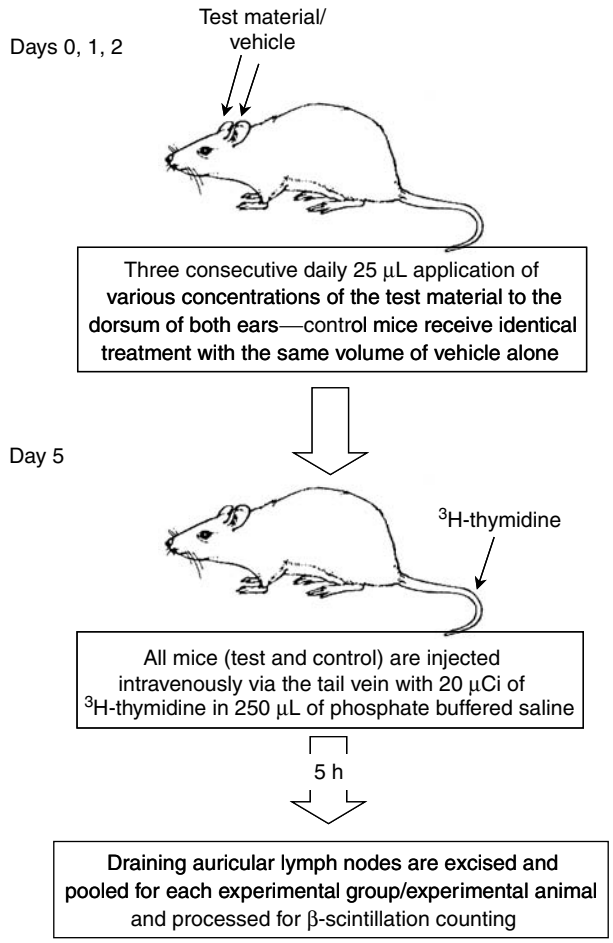


FIGURE 58.1 Conduct of the standard local lymph node assay.

protocol (De Jong et al., 2002; Ehling et al., 2005a,b; Homey et al., 1998; Ikarashi et al., 1993, 1994, 1996; Suda et al., 2002; Ulrich et al., 1998, 2001; Van Och et al., 2000). Such modifications of the standard assay in mice are in various stages of evolution and evaluation, although none has yet been validated formally as is the case with the standard method (see Section 58.3). Although some of these approaches may have some merit, a detailed commentary on all tests proposed as LLNA variants and modifications is beyond the scope of this article and they will not be considered here. Neither do we discuss here the merits or otherwise of conduct of the LLNA in species other than the mouse (Arts et al., 1996; Clottens et al., 1996; Ikarashi et al., 1992; Kashima et al., 1996; Maurer and Kimber, 1991).

58.3 EVALUATION AND VALIDATION

The LLNA was developed initially as a method for hazard identification. Although it is now clear that the LLNA is also of considerable utility in determination of relative potency and in the risk assessment process, it is for the purpose of hazard identification that the assay has been formally validated. That process of evaluation and validation is described here. Use of an LLNA for potency determinations and risk assessment is considered later.

The LLNA has been evaluated extensively in both national and international interlaboratory collaborative trials (Basketter et al., 1991, 1996; Kimber et al., 1991, 1995, 1998b; Kimber and Basketter, 1992; Loveless et al., 1996; Scholes et al., 1992), and has been the subject of searching comparisons with guinea pig predictive test methods and human sensitization data (Basketter and Scholes, 1992; Basketter et al., 1991, 1992, 1993, 1994; Kimber et al., 1990a, 1994; Ryan et al., 2000). Collectively, these investigations comprised analyses of a wide variety of chemicals. In addition, however, more discrete investigations of specific groups of materials have been conducted using either the standard LLNA, or modifications of it. Among these are studies of biocides (Basketter et al., 1999b; Botham et al., 1991; Hilton et al., 1998; Warbrick et al., 1999a), fragrance materials and materials used in personal care products (Hilton et al., 1996; Wright et al., 2001), metal salts (Basketter et al., 1999c), rubber chemicals (De Jong et al., 2002), petrochemicals (Edwards et al., 1994), dyes (Betts et al., 2005; Sailstad et al., 1994), and chemical mutagens and rodent carcinogens (Ashby et al., 1993; Warbrick et al., 2001; Wolfreys and Basketter, 2004). On the basis of these investigations and additional practical experience gained from the use of the method, the conclusion drawn was that the LLNA represented a viable alternative to guinea pig tests for the identification of contact allergens (Basketter et al., 1996; Chamberlain and Basketter, 1996; Gerberick et al., 2000).

Against this background, the LLNA was submitted in 1998 for consideration by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), an organization established in the United States by 14 federal regulatory and research agencies to harmonize the development, validation, and acceptance of new toxicological test methods (NIH, 1999). A peer review panel was appointed by ICCVAM and after intensive scrutiny of the method it concluded that, compared with other predictive tests, the LLNA offers advantages with respect to animal welfare (specifically in terms of refinement and reduction). The panel also recommended that the LLNA could be used as a stand-alone alternative for the purposes of hazard identification, subject to the implementation of certain protocol modifications. These proposed modifications included considerations of selection of mouse strain, the individual identification of mice, analysis of body weight changes, the use of statistical analyses, and the incorporation of a concurrent positive control (Dean et al., 2001; Haneke et al., 2001; NIH, 1999; Sailstad et al., 2001). The utility and application of these modifications have recently been the subject of a detailed commentary (Basketter et al., 2002), and a similar analysis here would be beyond the scope of this article. The important point is, however, that the LLNA was subjected to rigorous independent scrutiny and validated by ICCVAM as an appropriate method for hazard identification. There soon followed a similar endorsement by the European Centre for the Validation of Alternative Methods (ECVAM) (Balls and Hellsten, 2000). In the light of these developments, the current regulatory status of the LLNA is outlined briefly in the next section.

Although the LLNA has been shown in the context of the validation exercises summarized earlier, to have levels of sensitivity, selectivity, and overall accuracy comparable with, or better than, the commonly used guinea pig tests, questions are nevertheless raised about specific issues relating to test performance. Among these are the ability of the assay to detect metal allergens (and in particular nickel), and the prevalence of false-positive responses.

Nickel is a common human allergen. Although modest responses to nickel chloride and nickel sulfate can be elicited in mice (Gerberick et al., 1992; Kimber et al., 1990b), the consensus is that nickel salts usually fail to test positive in the LLNA (Kimber et al., 1994). However, it has also often proven difficult to elicit responses to nickel salts in guinea pig tests (Buehler, 1965; Goodwin et al., 1981; Wahlberg, 1989). The likelihood is that nickel is probably only a very weak allergen and that the high prevalence of sensitization in some societies is due to the ubiquitous distribution of this substance and extensive opportunities for exposure. The ability of the LLNA to detect metal salts, which are known to be implicated in allergic contact dermatitis, has now been examined systematically. Thirteen metal salts were studied, of which eight were considered to be contact allergens. The remaining five were considered not to cause skin sensitization. With the exception of nickel chloride, all known allergens (tin chloride, cobalt chloride, mercuric chloride, ammonium tetrachloroplatinate, potassium dichromate, beryllium sulfate, and gold chloride) were found to elicit positive responses in the LLNA (Basketter et al., 1999c). Of the five non-sensitizers, four (zinc sulfate, lead acetate, manganese chloride, and aluminium chloride) failed to induce positive LLNA responses, and only one (copper chloride) tested positive (Basketter et al., 1999c). Taken together, these data indicate that, in the majority of instances, the LLNA provides an accurate assessment of the likely skin sensitizing potential of metal salts. The argument is, however, rather academic given new metals (and therefore metal allergens) are unlikely to be discovered.

The other issue is the possibility of false-positive results. One apparent anomaly in the performance of the LLNA is the fact that sodium lauryl sulfate (SLS), a nonsensitizing skin irritant, has been shown by some investigators to elicit positive, albeit weak responses (Basketter et al., 1994; Kimber et al., 1994; Loveless et al., 1996; Montelius et al., 1994). It is possible that SLS may represent something of a special case, insofar as it is known that this chemical is able to cause the migration of epidermal LC to the skin draining lymph nodes (Cumberbatch et al., 1993), although the relevance of this for the initiation of LNC proliferative activity is not clear. Although certain skin irritants are able in some instances to provoke comparatively low level activity in draining lymph nodes, this does not necessarily compromise the correct interpretation of test data, or prevent the accurate identification of chemicals that have the potential to cause skin sensitization (Basketter et al., 1998). Moreover, it is important to appreciate that the majority of nonsensitizing skin irritants fail to elicit positive responses in the LLNA (Basketter et al., 1998; Gerberick et al., 1992; Kimber et al., 1991, 1995).

58.4 INTERNATIONAL REGULATORY STATUS OF THE LLNA

The adoption of a new test method into regulatory guidelines represents a substantial challenge, demanding both a general scientific consensus on its suitability, as well as acceptance via the formal processes prescribed for validation. As described earlier, this latter step was undertaken for the LLNA via ICCVAM. The report of this independent review has been published (NIH, 1999). ICCVAM concluded that the method was fully valid as a stand-alone alternative to, or replacement for, existing guinea pig tests. As a result, the LLNA was adopted by several federal regulatory agencies in the United States as an accepted method for skin sensitization testing. In addition, the LLNA has been incorporated into a new Test Guideline (No. 429; *Skin Sensitization: Local Lymph Node Assay*) by the Organization for Economic Cooperation and Development (OECD), and this was adopted formally in 2002 (OECD, 2002). Similarly, the European Union (EU) has prepared a new test method on the LLNA (B42); the text closely following that prepared by the OECD. As a reflection of these developments, the United Kingdom competent authority (ca; health and safety executive, HSE) in 2002 effected a change of policy with effect to skin sensitization. The guidance now provided to notifiers by the HSE indicates that the LLNA will be accepted as part of a notification under the *notification of new substances* (NONS) regulations. The statement issued by the HSE also stated

> The LLNA provides certain advantages with regard to animal welfare (most particularly refinement but also reduction) and also scientific aspects (such as the objective and quantitative nature of the end-point measured). The LLNA can also provide information on the relative potency of contact sensitizers, unlike other methods currently available for skin sensitization. Given these significant advantages the UK ca now considers that for notification purposes the LLNA is the method of first choice for skin sensitization.

Since that position was reached in 2002, the HSE has accumulated experience with the use in practice of the LLNA within a regulatory context. A retrospective analysis of LLNA study reports received since the foregoing statement was released has been conducted recently (Cockshott et al., 2006). One conclusion reached was that contrary to some concerns that the LLNA might prove to be either less sensitive or more sensitive than the guinea pig maximization test, the proportion of new substances notified under NONS and classified as skin sensitizers was comparable with previous data before introduction of the LLNA (Cockshott et al., 2006).

Finally, it is important to emphasize here that the LLNA was designed initially and developed as a method for assessment of the skin sensitization hazards of chemicals, rather than of complex mixtures or finished product formulations. It is for that purpose that the assay was evaluated and subsequently validated.

58.5 THE LLNA AND ASSESSMENT OF RELATIVE POTENCY

Although accurate identification of hazard is a required first step in any toxicological evaluation, it does not of itself necessarily inform the risk assessment process. What is really needed, in concert with an appreciation of likely conditions of exposure, is information regarding toxicological potency. With respect to the induction of skin sensitization, potency should be defined as a function of the amount of chemical that is necessary to induce sensitization in a previously naïve subject. Actually the important metric for skin sensitization is the amount of chemical per unit surface area of skin (e.g., $\mu g/cm^2$). In fact, the most compeling illustration of this in humans derives from volunteer studies conducted by Friedmann and colleagues. They were able to demonstrate that, in most circumstances at least, the acquisition of skin sensitization is critically dependent upon the amount of chemical experienced per unit area of skin (Friedmann, 1990). For some time, a major focus of attention has been on defining how the LLNA can be used to assess experimentally the relative sensitizing potency of contact allergens (Basketter et al., 2001a; Kimber et al., 2001; Kimber and Basketter, 1997).

The induction by chemical allergens of proliferative responses in draining lymph nodes not only provides a marker of skin sensitizing activity, but also a quantitative correlate of the extent of sensitization (Kimber et al., 1999; Kimber and Dearman, 1991). It is reasonable, therefore, to speculate that it should be possible to determine the relative potency of chemicals on the basis of the vigor of responses induced in the LLNA. For this purpose, an EC3 value is derived from dose-related activity in the LLNA; an EC3 value being defined as the amount of chemical (absolute amount of chemical or chemical per unit area, or percentage or molar concentration) that is required to induce in the assay a response of the magnitude that in practice defines skin sensitizing potential (an SI of 3).

Careful thought was given to the most suitable method for deriving EC3 values from LLNA dose responses. Investigations were conducted in which three possible approaches were compared: quadratic regression analysis, Richard's model, and simple linear interpolation. The conclusion drawn was that linear interpolation between values either side of the threefold SI on an LLNA dose–response curve provides the most robust and convenient method for calculation of EC3 values (Basketter et al., 1999d).

This approach can be expressed mathematically as

$$\mathrm{EC3} = c + \left[\frac{(3-d)}{(b-d)}\right] \times (a-c)$$

where (a,b) and (c,d) are the coordinates, respectively, of data points lying immediately above and immediately below the SI value of 3.

It could be argued that there are more sophisticated approaches available for interrogation of dose–response relationships and that the application of these might provide for greater accuracy. Although it might appear scientifically heretical to reject such methods in favor of the much more straightforward approach of linear extrapolation, as will become apparent, it is neither necessary nor helpful for classification purposes to measure with great accuracy small, and probably biologically insignificant differences between chemical allergens in terms of EC3 values.

Experience to date reveals that EC3 values are very robust parameters of LLNA responses, both with time within a single laboratory, and also between laboratories. Thus, for instance, it was found in studies of HCA conducted by a single laboratory over a 10-month period that EC3 values were very consistent, ranging from 6.9 to 9.6% (Dearman et al., 1998). Similar consistency was found when EC3 values for *p*-phenylenediamine (PPD) were measured each month over a 4-month period (Warbrick et al., 1999b). Consistency of derived EC3 values was reported also with sequential analyses of isoeugenol (Basketter and Cadby, 2004). The results of interlaboratory collaborative trials of the LLNA demonstrated that very similar EC3 values were derived when the same chemical was analyzed in several independent laboratories (Kimber et al., 1995, 1998b; Loveless et al., 1996; Warbrick et al., 1999b).

In practice, EC3 values have been used successfully to determine the relative skin sensitizing potency of several series of chemicals, including isothiazolinone biocides (Basketter et al., 1999b), dinitrohalobenzenes (Basketter et al., 1997), and various aldehydes (Basketter et al., 2001c).

The real test of the utility of relative potency measurements based on EC3 values is the extent to which they are congruent with what is known of the activity of sensitizing chemicals among human populations. To address this issue analyses were undertaken in partnership with clinical dermatologists who provided a view of the relative skin sensitizing potency of two series of known human contact allergens. Chemicals were classified according to relative potency based on clinical judgment and experience. These classifications were then compared with EC3 values derived for the same chemicals. In each of the two investigations, there was a very close correlation between clinical potency and EC3 values (Basketter et al., 2000; Gerberick et al., 2001b).

Based on these analyses, and other investigations, it is relevant to consider how measurement of relative skin sensitizing potency might be best exploited for the purposes of improved classification and labeling. The importance of this derives from the apparent wide variations in the potency. Thus, it is estimated that contact allergens vary by up to four or five orders of magnitude with respect to their relative skin sensitizing potency. This being the case, there is clearly merit for the purposes of risk assessment and risk management in distinguishing between allergens that vary significantly in activity.

This opportunity has been considered in some detail during the last few years (Basketter et al., 2005; Kimber et al., 2003; Schneider and Akkan, 2004). A view was that the most appropriate potency classifications would make use of the following descriptors: *extreme, strong, moderate, and weak*; with of course inactive chemicals being classified as nonsensitizers (Kimber et al., 2003).

One recommendation for equating these classifications to EC3 values used as the unit for the latter percentage of concentration (as follows). However, other metrics, such as those based on molar exposure concentrations, or on exposure per unit area of skin (μg of chemical/cm^2 of skin surface) would be equally legitimate. The proposed classification was: *extreme* (EC3 <0.1%); *strong* (EC3 ≥ 0.1 to <1); *moderate* (≥1 to <10), and *weak* (≥10 to ≤100) (Kimber et al., 2003). As indicated earlier, nonsensitizing chemicals would not have a measurable EC3 value because, by definition, they fail at all test concentrations to provoke a threefold or greater increase in LNC proliferation compared with vehicle controls (Gerberick et al., 2001b).

It must be emphasized that the preceding is only one possible classification scheme for grading contact allergens as a function of EC3 values. Nevertheless, it does have the merit of providing a rank order that correlates well with human experience of sensitizing potential.

Before considering how in practice the relative potency of contact allergens based on EC3 values can be integrated into the risk assessment process, it is necessary to address one point that was alluded to earlier; the relevance of vehicle matrix for relative potency. It is clear that the form in which a chemical is encountered at skin surfaces can impact upon the effectiveness with which contact sensitization is acquired, and this is of potential importance in establishing likely risks to human health. There is good evidence that the vehicle in which a chemical allergen is applied to the skin can have a significant influence on LLNA responses and EC3 values; the implication being that the vehicle may affect overall sensitizing potency (Basketter et al., 2001b; Lea et al., 1999; Warbrick et al., 1999a,b; Wright et al., 2001). Currently, it is not possible to draw any conclusions regarding which vehicles may potentiate skin sensitization. Indeed, such generalizations may not be possible as experience to date suggests that the impact of vehicle upon the effectiveness of sensitization will vary significantly according to the physicochemical characteristics and dose of the chemical allergen. Notwithstanding these uncertainties, there is every reason to conclude that the vehicle formulation can influence the induction of skin sensitization and that this is an important consideration when developing risk assessments.

58.6 INTEGRATION OF LLNA DATA INTO RISK ASSESSMENT

Skin sensitization risk assessment of new chemicals is a critical step before their introduction into the workplace or marketplace. The basic process used for evaluating the skin sensitization risk of a new product ingredient is to consider a no effect/safety factor approach. This is a stepwise approach that may involve analytical assessments, preclinical skin sensitization testing, clinical evaluation, and benchmarking of resulting data against similar ingredients or product types (Robinson et al., 1989; Gerberick et al., 1993; Kimber and Basketter, 1997; Basketter, 1998; Gerberick and Robinson, 2000). It is the potential for an adverse effect to occur in humans exposed during manufacturing or product use that is being determined. This approach incorporates assessment of both inherent toxicity and exposure to the new ingredient. Specifically, it involves determination of the likely extent of exposure to the test material (exposure assessment), and its sensitization potency (dose–response assessment). It is the ability of the LLNA to assess skin sensitization potency that makes it an invaluable tool for conducting sound, quantitative exposure-based risk assessments (Robinson et al., 2000; Gerberick et al., 2001a).

Despite the importance of potency estimation in the development of accurate risk assessments, there had previously been only relatively modest progress in the definition of appropriate experimental models. The standard guinea pig tests, such as the maximization test, were successful at hazard identification (Andersen and Maibach, 1985; Botham et al., 1991), and there has been some interest in the use of a modified guinea pig maximization test for consideration of relative potency. Of note has been the work of Andersen and coworkers, who manipulated the guinea pig maximization test to obtain dose–response data (Andersen et al., 1995). However, the LLNA provides new opportunities for the objective and quantitative estimation of skin sensitization potency (Kimber and Basketter, 1997; Dearman et al., 1999).

Experience to date with this approach has been encouraging; clear differences between skin sensitizing chemicals can be discerned, and such differences appear to correlate closely with the ability of the materials to induce contact allergy in experimental models, and with what is known of their sensitizing activity in humans (Hilton et al., 1998; Basketter et al., 1999b, 2000; Gerberick et al., 2001b). As discussed earlier, the LLNA is being used now in the development of classification schemes for ranking contact allergens according to potency and this will enable further refinement of risk assessment and risk management.

58.7 NEW DEVELOPMENTS

Two recent developments/initiatives merit brief mention here. The first of these is the compilation and wide availability of comprehensive datasets of the behavior of chemicals in the LLNA (Gerberick et al., 2004, 2005). It is anticipated that these databases, which comprise a unique archive of LLNA results and related information, will provide a very valuable resource for the evaluation of alternative approaches to skin sensitization testing, including novel *in vitro* methods, and methods based on considerations of structure–activity relationships (SAR).

The second initiative is the design of cut-down or reduced LLNA formats that might be of value in particular circumstances for the purpose of screening; perhaps, when there is a requirement for identification of skin sensitization hazards among large numbers of chemicals (Kimber et al., 2006). One particular option that is being discussed currently is the examination in a reduced LLNA of only a single concentration of the test chemical. However, there may, in addition, be opportunities to consider reductions in group size.

The advantage of such modified assays is that they offer potentially significant animal welfare benefits with respect to reduced usage. However, against this has to be balanced the fact that the use of only a single dose of the test chemical would in most circumstances prohibit derivation of an EC3 value and determination of relative potency. It is therefore the case that cut-down versions of the LLNA will have utility only in circumstances where the primary objective is hazard identification, rather than measurement of relative potency or risk assessment (Kimber et al., 2006).

58.8 CONCLUSIONS

The LLNA is now of proven value for the purposes of skin sensitization hazard identification. It has been formally validated in this respect and has been accepted broadly in regulatory guidelines. It has been also acknowledged that the assay provides important animal benefits. Fewer animals are needed and animals are subject to reduced trauma and discomfort.

Moreover, it is now acknowledged that the LLNA provides a coherent approach to defining relative potency as an important contribution to the risk assessment process.

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59 Iontophoresis in Humans: Regional Variations in Skin Barrier Function and Cutaneous Irritation

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59.1 INTRODUCTION

Iontophoresis is a process, which enhances transport of ionized substances into or through a tissue by the application of low level electrical potential [1,2]. *In vitro* studies [3–5] and *in vivo* animal studies [6,7] have demonstrated the feasibility of this method for enhancing drug penetration and absorption into systemic circulation. This approach seems to be promising for transdermal delivery of large and poorly permeable drugs. Currently, iontophoretic delivery systems for lidocaine (LidoSite™, developed by Vyteris, Inc.) and fentanyl (IONSYS™, developed by Alza Corp.) were approved by Food and Drug Administration (FDA) for human use. Furthermore, these promising drug-delivery systems can be potentially adapted and customized to administer peptide and protein drugs.

Transdermal iontophoresis involves the application of mild electric current either directly to the skin or indirectly through the drug formulation. The enhancement of the transport of poorly permeable molecules across the skin is attributed primarily to electrorepulsion and electroosmosis and is considered to be a safe procedure. Iontophoretic parameters that affect the skin safety include current intensity, length of application, electrode type in addition to pH of the formulation, permeant type, region of administration, and ethnicity [1,8]. The maximum exploitation of this delivery route will depend on the understanding and the ability to manipulate biological aspects of the interface between systems and the skin [9]. Iontophoresis is being investigated as a method of drug delivery by the application of modern electronics, material science, and skin toxicology [10]. Despite all these developments, there are concerns about issues regarding the effects of iontophoresis on skin safety in humans, including skin barrier perturbation and irritation.

Skin irritation covers many manifestations of provoked nonimmunologic cutaneous responses in living tissue [11] by application of stimuli. The outermost layer of skin, the stratum corneum, is regarded as the primary barrier to the external environment. The stratum corneum also acts as a barrier to avoid the loss of internal body components, particularly water [12]. Irritation tends to reduce the efficiency of stratum corneum barrier function and results in an increase in transepidermal water loss (TEWL). Hence, TEWL is regarded as an indicator of skin barrier function as high TEWL generally indicates barrier perturbation [13,14]. TEWL has been used in relation to the assessment of either the effects of penetration enhancers [15] or the irritation [16] on the skin. This is sometimes associated with a decrease in skin water content [17] and increase in skin temperature [18]. Hence, measurement of skin capacitance or skin hydration [19] and skin temperature may also be used to assess irritation [20,21].

Percutaneous absorption in humans varies with the region on which the drug is applied [22]. A high absorbing area may be desirable to deliver sufficient drug for systemic effect through the transdermal route. Feldman and Maibach [23]

explored the potential for regional variation in percutaneous absorption with hydrocortisone and pesticides in man [24]. Rougier et al. [25] examined the influence of anatomical site on the relationship between total penetration of a drug in man and the quantity present in the stratum corneum 30 min after application. Therefore, understanding on the regional differences on skin barrier function and skin irritation in response to iontophoresis is needed to consider a suitable body site for iontophoretic delivery of drugs.

The application of mild electric current may leave the target tissue damaged depending on the electrical parameters associated with iontophoresis. Hence, it is important to understand the skin barrier function and irritation following iontophoresis. The acceptance and further development of this technique depends on ensuring that it does not provoke unacceptable side effects and the skin barrier integrity is not compromised. This chapter reviews the skin safety studies in humans, addressing regional skin barrier function and cutaneous irritation, using transdermal iontophoresis.

59.2 GENERAL METHODOLOGY

In 1944, Draize et al. [26] developed a dermatological evaluation system of visual scoring for measuring erythema and edema, which remains standard. Currently, several methods are available, such as TEWL for barrier integrity; skin capacitance for moisture content; impedance spectroscopy for skin impedance; laser Doppler flowmetry for cutaneous blood flow; and ultrasound scan for skin thickness [27,28], to study skin barrier properties and irritation in a noninvasive way. In searching for sophisticated assessment of subtle nonvisible and palpatory changes, skin bioengineering methods have been used [29–31] after saline iontophoresis for 10 min using saline solutions to evaluate methods of assessing skin integrity. The above investigated the effect of two iontophoresis treatment conditions on skin integrity and measured several responses (i.e., TEWL, skin capacitance, skin temperature, skin color, and primary skin irritation via the visual scoring system). The findings suggested that the experimental methodology was useful for assessing changes in skin integrity resulting from saline iontophoresis.

TEWL offers the possibility to monitor the skin barrier function *in vivo*. TEWL can be measured quantitatively using a Tewameter™ (Courage & Khazaka, Cologne, FRG) or Evaporimeter™ (Servo Med, Stockholm, Sweden). According to Fick's first law, TEWL allows determination of the water vapor gradient established from the deepest skin layers to the outer environment [32].

The measuring principle of the water evaporation is based on the diffusion in an open chamber:

$$dm/dt = (-D)(A)\,dp/dx$$

where

m = water transported (in g)
t = time (h)
D = diffusion constant
A = surface area in m^2
p = vapor pressure of the atmosphere (mmHg)
x = distance from skin surface to point of measurement

Skin capacitance measurement provides a method to determine the hydration level of the skin surface. The electrical capacitance of the skin surface can be measured using Corneometer™ (Courage & Khazaka, Cologne, FRG). This device, described in detail [33], indirectly measures the water content of the stratum corneum. The measuring principle is based on capacitance measurement of a dielectric medium. Any change in the dielectric constant due to skin surface hydration variation alters the capacitance of a precision measuring capacitor.

Electrical impedance spectroscopy, a noninvasive biophysical tool [34], has been used to evaluate the electrical properties of the skin [35–37]. The stratum corneum is composed of approximately 100 lipid bilayers with embedded corneocytes and is relatively nonconductive with high impedance [38]. The impedance of the skin can be measured by applying a voltage in the frequency ranges of about 1 mHz–1 MHz. The technique can be used to assess the skin integrity and skin barrier recovery following iontophoresis.

The visual scoring system, to evaluate primary skin irritation, described by Draize et al. [26] is a standard way to grade cutaneous erythema (on a scale of 1–4): very slight erythema barely perceptible #1; well-defined erythema #2; moderate to severe erythema #3; severe erythema, beet redness to slight eschar formation, injuries in depth #4; and edema (on a scale of 1–4): very slight edema, barely perceptible #1; slight edema, edges of area well defined by definite raising #2; moderate edema, area raised approximately 1 mm #3; severe edema, raised more than 1 mm, and extending beyond the area of exposure #4.

A number of variables affect these measurements. To work under standardized conditions is of the utmost importance to obtain reliable and reproducible results. Environmental conditions should be closely monitored for room temperature and relative humidity. TEWL has been shown to vary directly with skin temperature [18,39]. With the skin barrier perturbation, the skin temperature can vary and skin temperature can be measured with an infrared thermometer.

59.3 SKIN BARRIER FUNCTION

The barrier property of the skin is critical in preventing the entry of exogenous toxic chemicals/microbes into the body and also to avoid the loss of internal body components, particularly water [12]. The outermost layer of skin, stratum corneum, plays a dual role by providing barrier to TEWL and the entry of exogenous substances. This section of the chapter describes the *in vivo* human studies monitoring skin barrier property due to iontophoresis.

59.3.1 Transepidermal water loss

TEWL is the rate at which water vapor is lost from the body across the skin. Measurement of TEWL is a powerful

noninvasive method that is widely used to characterize the macroscopic changes in the skin barrier properties [40]. When the skin is damaged by physical or chemical means, the skin barrier property is compromised with an increase in TEWL values. TEWL is a well-accepted method for quantifying material innocuousness [41] and provides robust method for assessing damage to the stratum corneum. TEWL has been used in relation to the assessment of either the effects of penetration enhancers [15,42] or the irritation [13,31,43,44] on the skin. Irritation tends to reduce the efficiency of stratum corneum barrier function and results in an increase in TEWL.

The effect of iontophoresis on TEWL in humans has been reported in several studies [13,31,36,45,46]. The TEWL values were measured before iontophoresis (baseline levels) and after termination of iontophoresis. Skin barrier changes due to brief clinical applications of iontophoresis with low-voltage electrodes are generally reported to be mild [31] and occur more frequently after treatments, which are short (10–20 min) but of relatively high current densities (e.g., 1.3 mA/cm^2). Van der Geest et al. [36] reported that there was no effect on TEWL by application of either 0.25 mA/cm^2 constant current or 0.50 mA/cm^2 pulsed continuous current (100 Hz, 50% duty cycle) for 30 min compared to the control (no current). The enhanced TEWL values immediately following the patch removal in both iontophoresis and control subjects is thought to be the result of occlusion of skin by the patch itself.

The human skin displays remarkable regional variation in percutaneous absorption of different molecules [23,47]. In another study of regional variations in skin barrier due to iontophoresis, Singh et al. [13] reported that the TEWL values at abdominal site were increased compared to baseline levels after 4 h of iontophoresis using 0.2 mA/cm^2 current density. Table 59.1 shows that the TEWL values were significantly higher 1 h after patch removal both at the anode ($p < .001$) and the cathode ($p < .05$), which returned to baseline after 2 h of patch removal ($p > .05$). Repeated measure analysis for response variable showed a significant difference ($p < .01$) in the mean TEWL due to iontophoresis at 1 h after patch removal, which resolved at 2 h of evaluation. However, the study reported that there was no significant difference in TEWL ($p > .05$) either at the anode or the cathode at upper arm and chest. The application of iontophoresis (0.2 mA/cm^2) for 4 h in different ethnic groups did not produce any clinically relevant changes either at anode or cathode indicating the safety of the procedure [45]. A recent study also reported that the transdermal iontophoresis induced a significant increase in TEWL at both the anodal and cathodal sites [46]. It was demonstrated by the test of repeated measurements multiway ANOVA that the enhancement in TEWL was mainly due to the occlusion of formulation solution in the electrode chambers, and only very slightly by the current application to the skin.

59.3.2 Skin Capacitance

Skin capacitance is a measure of the water content from skin surface to the deeper layers (20–40 μm) including the stratum corneum and partial viable epidermis [48]. Although it is a measure of the water content of the skin, it is only an indirect measure of barrier function. TEWL allows determination of the water vapor gradient established from the deepest skin layers to the outer environment and skin capacitance is a measure of the water content from skin surface to the deeper layers. Therefore, TEWL measurements and skin capacitance should correlate well in normal skin. In contrast, pathological skin such as scaly or psoriatic skin does not show such a correlation between TEWL and skin conductance [49].

Skin capacitance was reported to be elevated at 15 min after the iontophoretic patch removal compared to baseline levels, which recovered to baseline levels at 60 min [31]. The baseline skin capacitance was found to vary significantly ($p < .05$) at various sites on the body [13]. It was also reported that there was a significant decrease in capacitance at the abdomen due to iontophoresis at the anode ($p < .05$) 2 h after patch removal and an increase at the cathode ($p < .01$) 1 h after patch removal while there was no significant effect of iontophoresis on capacitance at the upper arm under the anode and the cathode ($p > .05$). Table 59.2 shows the effect of iontophoresis on skin capacitance at the abdomen site. Capacitance was significantly

TABLE 59.1
Effect of Iontophoresis on TEWL at the Abdomen Site

	TEWL (g/m^2/h), Mean ± SE				*p* Value	
	Anode		Cathode		Test of Difference from Baseline	
Time[a] (min)	S[b]	B[b]	S[b]	B[b]	Anode	Cathode
−10	5.14 ± 3.56	5.36 ± 4.28	4.85 ± 5.70	5.07 ± 7.75		
60	5.65 ± 3.16	4.81 ± 2.46	4.10 ± 3.18	3.91 ± 3.16	0.0009	0.04
120	5.64 ± 2.00	5.43 ± 3.49	4.87 ± 3.72	5.04 ± 3.00	0.15	0.68

Source: Singh, J., Gross, M., Sage, B., Davis, H.T., and Maibach, H.I., *Food Chem. Toxicol.*, 39(11), 1079–1086, 2001. With permission.

[a] Time: –10 min is measurement taken before iontophoresis (baseline).

[b] S, signal; B, background.

TABLE 59.2
Effect of Iontophoresis on Skin Capacitance at the Abdomen Site

	Capacitance (i.u.), Mean ± SE				*p*-Value	
	Anode		Cathode		Test of Difference from Baseline	
Time[a] (min)	S[b]	B[b]	S[b]	B[b]	Anode	Cathode
–10	129.60 ± 9.40	129.85 ± 9.06	128.50 ± 13.50	128.33 ± 13.49		
60	122.00 ± 5.86	123.00 ± 4.85	129.53 ± 6.63	126.98 ± 6.86	0.63	0.01
120	119.25 ± 5.07	123.13 ± 6.78	126.18 ± 6.93	125.55 ± 6.42	0.02	0.64

Source: Singh, J., Gross, M., Sage, B., Davis, H.T., and Maibach, H.I., *Food Chem. Toxicol*., 39(11), 1079–1086, 2001. With permission.

[a] Time: –10 min is measurement taken before iontophoresis (baseline).

[b] S, signal; B, background.

TABLE 59.3
Effect of Iontophoresis on Skin Temperature at the Upper Arm Site

	Temperature (°C), Mean ± SE				*p*-Value	
	Anode		Cathode		Test of Difference from Baseline	
Time[a] (min)	S[b]	B[b]	S[b]	B[b]	Anode	Cathode
−10	31.85 ± 0.87	31.75 ± 1.86	31.80 ± 2.16	31.80 ± 1.88		
0	32.08 ± 0.32	31.48 ± 0.42	32.28 ± 0.36	31.70 ± 0.47	0.005	0.001
60	33.08 ± 0.60	32.38 ± 0.59	32.93 ± 0.75	32.40 ± 0.74	0.007	0.003
120	33.25 ± 0.49	32.68 ± 0.60	33.15 ± 0.73	32.70 ± 0.64	0.01	0.005

Source: Singh, J., Gross, M., Sage, B., Davis, H.T., and Maibach, H.I., *Food Chem. Toxicol*., 39(11), 1079–1086, 2001. With permission.

[a] Time: –10 min is measurement taken before iontophoresis (baseline).

[b] S, signal; B, background.

lower ($p < 0.05$) at the chest under the anode 1 h after patch removal [13].

59.3.3 Skin Temperature

The changes in skin temperature provide an indication of barrier perturbation and cutaneous irritation. Camel et al. [31] reported that the skin temperature was slightly elevated at both anode and cathode sites following iontophoresis compared to control sites. The application of electrical current to skin may result in Joule heating due to the electrically resistive nature of skin. However, the study noted that extremely low levels of current used in iontophoresis alone may unlikely result in Joule heating and the increased blood flow due to erythema may be the most likely cause. In a different study, Singh et al. [13] examined regional variations and reported a significant increase ($p < .05$) of skin temperature at 1 and 2 h following iontophoresis at the anode and the cathode of the upper arm (Table 59.3). However, there was no significant increase in skin temperature ($p > .05$) at the abdomen site at all observation time points except 2 h after patch removal at the anode ($p < .05$). At the chest site, there was no significant effect of iontophoresis ($p > .05$) on skin temperature except 1 h after patch removal at the anode ($p < .01$) and immediately after patch removal at the cathode ($p < .05$).

59.3.4 Skin Impedance

Impedance spectroscopy quantitatively evaluates the electrical properties of the barrier. Electrical impedance in healthy human volunteers at five different skin sites was measured [50]. Significant differences in impedance values among different anatomical regions of normal skin were found. Yamamoto and Yamamoto [51] have demonstrated *in vivo* that the skin's electrical resistance resides primarily in the stratum corneum with skin impedance decreasing as layers of the stratum corneum were sequentially removed by tape stripping. Normal human skin has relatively high impedance and if the skin barrier is compromised, the skin becomes more permeable to the passage of ions into and across the stratum corneum thereby leading to a decrease in impedance [38,52]. Kalia and Guy [35] investigated the effects of iontophoresis on the electrical characteristics of human skin *in vivo*. The influence of iontophoretic current density, duration of current application, and hydration were studied. The authors observed that increasing both current

density and time of application caused a greater impedance drop and delayed the postiontophoretic recovery. In another study, Curdy et al. [37] investigated the effect of metal ions (Na^+, K^+, Ca^{2+}, and Mg^{2+}) on the recovery of skin impedance following iontophoresis. No differences were observed in the presence of different ions at two different ionic strengths. The skin impedance is known to vary from subject to subject and body site to site [13,38,50].

59.4 CUTANEOUS IRRITATION

Irritation due to brief clinical applications of iontophoresis with low voltage electrodes is generally reported to be mild [31]. Skin irritation is the observed response (erythema, edema, pain, itching, and heat) at the delivery or contact site. The skin irritation mechanism involves the release of inflammatory mediators and their migration to the exposed area. When the inflammatory mediators enter extracellular fluids, vasodilation results and cause visible erythema and increased vascular permeability that leads to edema.

59.4.1 Erythema and Edema

Erythema is the redness of the skin caused by increased blood flow to the capillaries. Iontophoresis increases cutaneous blood flow as measured by laser Doppler flowmetry and this increase reversible within 1 h is more pronounced at higher current density [53]. Iontophoresis is known to cause reddening of the skin due to the application of current [54]. Erythema may result from microscopic cellular damage at sites of high current density leading to cytokine and prostaglandin release and local vasodilatation [54]. The possibility of direct electric stimulation of erythema also exists [55]. Cutaneous stimuli can provoke release of substance P and calcitonin gene-related peptide (CGRP) at nerve endings in the epidermis [56,57]. CGRP is a more potent vasodilator, being responsible for a localized erythema lasting several hours. Its release may therefore be a reason for the erythematous responses seen with iontophoresis at all body sites.

Subclinical responses of skin erythema were reported following iontophoresis at different current densities for 10 min [31]. Skin reddening was also observed by measuring skin color using chromameter. There were variations in the color observed at anode and cathode sites. The reddening of skin was modest subclinical and reduced by 60 min following termination of iontophoresis. A correlation of visual score for erythema and skin color measurement was reported even though the response was subclinical. In the same study, no edema was observed under similar treatment conditions indicating that iontophoresis is a mild procedure.

Erythema as a reaction to iontophoresis was observed at all the body sites [58]. Iontophoresis is shown to induce significantly higher erythema both at the anode and the cathode ($p < .01$) at the abdomen, upper arm, and chest, but these scores resolved 24 h after patch removal except at the chest under the anode [13]. Thus, the chest was more sensitive because the erythema score was greater than at the upper

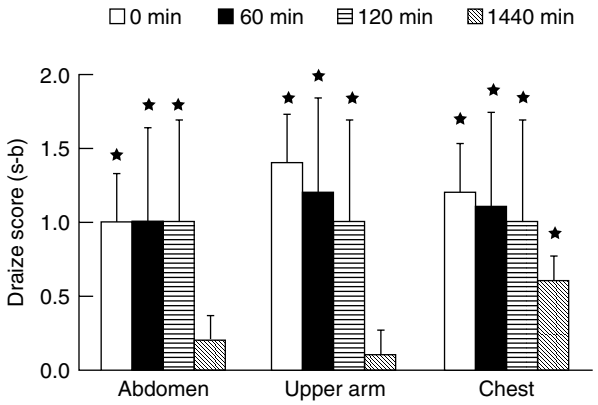


FIGURE 59.1 Effect of iontophoresis on visual scores at the anode sites. Values plotted are the mean $[(vs_{at}–vs_{ct})–(vs_{a0}–vs_{c0})]$ for each time t after termination of iontophoresis, where vs_{at} and vs_{ct} are the measured visual scores at the active and control sites at time t, respectively. These contrasts of measurements recorded at time t $(vs_{at}–vs_{ct})$, with the baseline measurements $(vs_{a0}–vs_{c0})$, define the iontophoresis effect on visual scores at each time t after termination of iontophoresis. "★" indicates statistical significance ($p < 0.05$) in comparison to the background. (Singh, J., Gross, M., Sage, B., Davis, H.T., and Maibach, H.I., *Food Chem. Toxicol.*, 39(11), 1079–1086, 2001. With permission.)

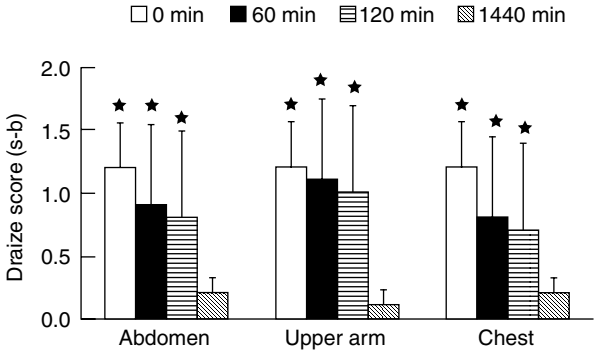


FIGURE 59.2 Effect of iontophoresis on visual scores at the cathode sites. Values plotted are the mean $[(vs_{at}–vs_{ct})–(vs_{a0}–vs_{c0})]$ for each time t after termination of iontophoresis, where vs_{at} and vs_{ct} are the measured visual scores at the active and control sites at time t, respectively. These contrasts of measurements recorded at time t $(vs_{at}–vs_{ct})$, with the baseline measurements $(vs_{a0}–vs_{c0})$, define the iontophoresis effect on visual scores at each time t after termination of iontophoresis. "★" indicates statistical significance ($p < 0.05$) in comparison to the background. (Singh, J., Gross, M., Sage, B., Davis, H.T., and Maibach, H.I., *Food Chem. Toxicol.*, 39(11), 1079–1086, 2001. With permission.)

arm or abdomen after 24 h of the termination of iontophoresis. Figures 59.1 and 59.2 show the effect of iontophoresis on Draize scores for erythema at different sites of the body under anode and cathode, respectively. There was no edema at any of the studied body sites. It was also observed that the erythema scores were modestly elevated due to iontophoresis in comparison to baseline in four ethnic groups (e.g., Caucasians, blacks, Hispanics, and Asians) at volar forearms; erythema resolved within 24 h [45]. A recent investigation by

Li et al. [46] reported that iontophoreis induced slight erythema and edema (Draize score of 1) compared with the control. Compared with the iontophoresis alone, the pretreatment of skin with a surfactant formulation (laureth-3 ethyloxylene ether, laureth-7 ethyloxylene ether, and sodium sulfosuccinate in a molar ratio of 0.7:0.3:0.05) caused slightly more skin erythema and edema (Draize score of 1 or 2) but did not further disturb the skin barrier function [46]. This study also emphasizes that skin irritation can be affected not only by iontophoretic parameters but also by formulation components.

59.4.2 Skin Reactions

In addition to the above mentioned changes observed in the skin, application of current may result in other skin reactions such as papules, rashes, tingling/warm sensations, and others. Papules are very small fluid-filled elevations on the skin and may be caused by the release of histamine from dermal mast cells; localized dermal cellular infiltrates or localized hyperplasia of dermal or epidermal cellular elements. In some individuals, application of current can cause increased redness, as well as the release of histamine in the skin and this can lead to the appearance of an allergic reaction even though the patient is not allergic to the drugs used. It is shown that iontophoresis, even at low current density, results in skin papules [13,31,45]. These skin reactions depend on the type of skin, site on the body in addition to the iontophoresis parameters. Unusual skin reactions were reported in a subject following iontophoresis with skin rash developed on day 2 following iontophoresis with dotty-like lesions by day 4 [46]. The reactions disappeared by day 10 without any treatment, but the exact nature of the skin reaction was unknown.

Iontophoresis in humans induced papules at different body sites such as abdomen, chest, and upper arm [13]. Papules were observed at the iontophoretically treated anode site and did not resolve in majority of subjects at the chest even after 24 h of termination of iontophoresis. At the most of sites, papules were transient after 24 h of iontophoresis, indicating that the electrical current induced short term, transient changes in the skin structure.

59.5 CONCLUSIONS

Iontophoresis-based drug-delivery systems are promising as shown by recent FDA approval of iontophoretic systems for lidocaine and fentanyl. These approvals demonstrate the overall safety of iontophoresis process in humans. The transdermal iontophoresis reversibly affects the skin barrier function and induces transient mild skin irritation, but does not cause any permanent damage to the skin at the current densities used. The effect of iontophoresis on skin barrier function and cutaneous irritation depends on the site of application on the body, type of skin (ethnicity), sensitivity of the skin in addition to the iontophoretic parameters and formulation composition. There is regional variation in the skin barrier functions and skin irritations due to iontophoresis in humans. Erythema and papules have been found to be greater at the chest than the abdomen or upper arm. Therefore, the chest would not be a preferred site over abdomen or upper arm for iontophoresis in humans.

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60 Contact Urticaria and Contact Urticaria Syndrome (Immediate Contact Reactions)

Smita Amin, Arto Lahti, and Howard I. Maibach

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60.1 INTRODUCTION

The contact urticaria syndrome (CUS) (immediate contact reactions) comprises a heterogeneous group of inflammatory reactions that appear usually within minutes after contact with the eliciting substance. They include not only wheal and flare but also transient erythema and may lead to eczema.

The epidemiology of these reactions is inadequately documented. The first such studies were performed in Hawaii (Elpern, 1985a, b, 1986), Poland (Rudzki et al., 1985), Sweden (Nilsson, 1985), Denmark (Veien et al., 1987), Finland (Turjanmaa, 1987), and Switzerland (Weissenbach et al., 1988). These studies suggested that immediate contact reactions are common in dermatologic practice. Some substances cause immediate reactions in almost everyone at the first contact (methyl nicotinate), but others need a period of sensitization (latex rubber).

Since the original description of the CUS in 1975 (Maibach and Johnson, 1975), new cases are published with increasing frequency.

60.2 SYMPTOMS

Immediate contact reactions appear on normal or eczematous skin within minutes to an hour or so, after agents capable of producing this type of reaction have been in contact with the skin. They disappear within 24 h, usually within a few hours. The symptoms can be classified according to morphology and severity: itching, tingling, or burning accompanied by erythema are the weakest type of immediate contact reactions, and are often produced by cosmetics (Emmons and Marks, 1985) and fruits and vegetables. Local wheal and flare is the prototype reaction of contact urticaria. Generalized urticaria after a local contact is uncommon. Tiny vesicles may rapidly appear on the fingers in protein contact dermatitis. Apart from the skin, effects may also appear in other organs in cases of strong hypersensitivity, thus leading us to neologize the term called CUS. In some cases, immediate contact reactions can be demonstrated only on slightly or previously affected skin, and it can be part of the mechanism responsible for maintenance of chronic eczemas (Hannuksela, 1980; Maibach, 1976; Veien et al., 1987).

There has been confusion in using terms such as contact urticaria, immediate contact reactions, atopic contact dermatitis, and protein contact dermatitis (Table 60.1). Immediate contact urticaria (ICU) includes both urticaria and other reactions, whereas protein contact dermatitis means allergic or nonallergic eczematous dermatitis caused by proteins or proteinaceous materials.

60.3 ETIOLOGY AND MECHANISMS

The mechanisms underlying contact reactions are divided into two main types, namely immunologic (immunoglobin E [IgE] mediated) and nonimmunologic immediate contact reactions (Lahti and Maibach, 1987). However, there are substances causing immediate contact reactions whose mechanism (immunologic or not) remains unknown.

Tables 60.2 through 60.4 present agents that have been reported to cause immediate contact reactions. They include chemicals in medications, industrial contactants, components of cosmetic products and of foods and drinks, and chemically undefined environmental agents. The pathogenetic classification (nonimmunologic versus immunologic) is also given but in many instances it is arbitrary, because the mechanisms of various contact reactions are unclear or mainly because a pathogenic evaluation was not performed.

Increasing awareness of immediate contact reactions will expand the list of etiologic agents, and more thorough understanding of pathophysiologic mechanisms will lead to a better and more rational classification of these reactions than at present. The international epidemic of latex protein contact urticaria has led to an awareness of the syndrome among surgeons, anesthesiologists, pediatricians, and gynecologists.

TABLE 60.1
Terminology of Contact Urticaria Syndrome

Terms	Remarks
Immediate contact reaction	Includes urticarial, eczematous, and other immediate reactions
Contact urticaria	Allergic (type I) and nonallergic (type II) contact urticaria reactions
Protein contact dermatitis	Allergic or nonallergic eczematous reactions caused by proteins or proteinaceous material

TABLE 60.2
Substances that have Caused Local Reactions and Anaphylactic Symptoms in Skin Tests

Aminophenazone
Ampicillin
Balsam of Peru
Bacitracin
Chloramphenicol
Diethyltoluamide
Egg
Epoxy resin
Latex protein (rubber products)
Mechlorethamine
Neomycin
Penicillin
Streptomycin

60.3.1 Immediate Contact Urticaria, IgE-Mediated Contact Reactions (Type I)

Immunologic contact urticaria reactions are immediate reactions in people who have previously become sensitized to the causative agent. In some cases of immunologic contact reaction, the respiratory, gastrointestinal, and genital tracts may have been the routes of sensitization. However, natural latex and some foods can sensitize people through the skin.

In skin challenge, the molecules of a contact reactant penetrate the epidermis and react with specific IgE molecules attached to mast-cell membranes. Cutaneous symptoms (erythema and edema) are elicited by vasoactive substances, mainly histamine released from mast cells. The role of histamine is important, but other mediators of inflammation, such as prostaglandins, leukotrienes, and kinins, may also influence the intensity of response. However, little is known regarding the dynamics of their interplay in clinical situations. More is known about the mediators of nonimmunologic contact urticaria (NICU).

Not only do mast cells and circulating basophils have Fc receptors for IgE molecules, but also eosinophils (Capron et al., 1981), peripheral B and T lymphocytes (Yodoi and Iskizaka, 1979), platelets (Joseph et al., 1983), monocytes (Melewicz and Spiegelberg, 1980), and alveolar macrophages (Joseph et al., 1980) can bind IgE. These findings make the issue of immunologic contact urticaria (ICU) more complicated than was believed earlier.

Patients with atopic dermatitis, but not other atopics or normal controls, have IgE on their epidermal Langerhans cells (Barker et al., 1988; Bruynzeel-Koomen, 1986; Bruynzeel-Koomen et al., 1986). This finding may provide an explanation for the high frequency of positive patch-test reactions to inhalant allergens, such as house dust mites, birch and grass pollen, and animal danders, in these patients (Adinoff et al., 1988; Leung et al., 1987; Mitchell et al., 1986; Reitamo et al., 1986; Tigalonowa et al., 1988). An important function of epidermal Langerhans cells is antigen presentation in delayed-type contact allergic reaction, but it can be hypothesized that protein allergens (inhalant, food, etc.) for type I immediate contact reactions bind to specific IgE molecules present on epidermal Langerhans cells, which become apposed to mononuclear cells (Najem and Hull, 1989) and induce a delayed-type hypersensitivity reaction resulting in eczematous skin lesions. This may be the mechanism whereby repeated immediate contact reactions lead to more persistent eczematous skin lesions.

Contact urticaria to rubber latex is a typical example of immediate immunologic contact reaction and is common (Estlander et al., 1987; Pecquet and Leynadier, 1993; Turjanmaa, 1987; Turjanmaa and Reunala, 1988; Wrangsjo

et al., 1986). Anaphylactic symptoms and generalized urticaria have occurred after contact with surgical (Axelsson et al., 1987; Carrillo et al., 1986; Spaner et al., 1989; Turjanmaa et al., 1988a) and household rubber gloves (Seifert et al., 1987). These reactions have been shown to be immediate, allergic, and IgE-mediated (Frosch et al., 1986; Seifert et al., 1987; Turjanmaa and Reunala, 1989; Turjanmaa et al., 1989). The allergens are among the proteins that constitute 1–2% of natural latex. Allergy to latex can be established by open application, skin prick tests (Turjanmaa et al., 1988c), and the latex radio-allergo-sorbent test (RAST) (Turjanmaa et al., 1988b).

Veterinary surgeons can contract contact urticaria on the hand after contact with cow amnion fluid, but they do not acquire reactions to cow dander in clinical provocation tests or in skin prick tests with cow epithelium extracts. RAST investigations have shown that antibodies to cow amnion fluid and serum, but not to epithelia, can be found in the sera of veterinary surgeons. The allergen causing contact urticaria in these cases is a compound of amnion fluid and serum but not of the epithelium of cows (Kalveram et al., 1986).

Foods are the most common causes of immediate allergic contact reactions (Table 60.3). The orolaryngeal area is a site where immediate reactions are provoked by food allergens, frequently among atopic individuals. Of 230 patients allergic to birch pollen, 152 (66%) gave a history of itching, tingling, or edema of the lips and tongue, and hoarseness or irritation of the throat when eating raw fruits and vegetables such as apple, potato, carrot, and tomato (Hannuksela and Lahti, 1977). Plum, peach, cherry, kiwi, celery, and parsnip can also elicit immediate contact reactions in birch pollen-allergic people. Positive results ("scratch-chamber" test) with suspected raw fruits and vegetables were noted in 36% of 230 patients. Apple, carrot, parsnip, and potato elicited reactions more often than swede (rutabaga), tomato, onion, celery, and parsley. The clinical relevance of the skin test results with apple, potato, and carrot was 80–90%. Only 7 of 158 (4%) atopic patients who were not allergic to birch pollen had positive skin test reactions to any of the fruits and vegetables.

RAST and RAST inhibition studies have confirmed the cross-allergy between birch pollen and fruits and vegetables. All immunological determinants in apple, carrot, and celery tuber appeared to be present also in birch pollen but not *vice versa* (Halmepuro and Løvenstein, 1985; Halmepuro et al., 1984).

60.3.2 Protein Contact Dermatitis

The term "protein contact dermatitis" was introduced (Hjorth and Roed-Petersen, 1976) for people with hand eczema demonstrating immediate symptoms when the skin was exposed to certain food proteins. Most of these individuals handled job-related food products for a protracted period before the symptoms appeared. Itching, erythema, urticarial swelling, or small vesicles appear on fingers or dorsa of hands within 30 min of contact with fish or shellfish. Wheat flour (causing baker's dermatitis) and natural rubber proteins are other examples of immediate contact reactions. Protein contact dermatitis may appear without previous urticarial rashes, but it may also be a result of repeated contact urticaria (Hannuksela, 1986). It is probable that both immunologic and nonimmunologic (irritant) types of protein contact reactions exist. Eczematous reactions are indistinguishable from primary irritant or allergic dermatitis, and careful study of the patient's history and the performance of skin tests ensure correct diagnosis. Awareness of these reactions provides profound relief for some hand eczema patients (Menné and Maibach, 1994; Turjanmaa, 1994).

60.3.3 Nonimmunologic Contact Urticaria

NICU occurs without previous sensitization and is the most common type of immediate contact reaction. The reaction remains localized and does not spread to become generalized urticaria, nor does it cause systemic symptoms. Typically, the strength of the reaction varies from erythema to an urticarial response depending on the concentration, the skin area exposed, the mode of exposure, and the substance itself (Lahti, 1980).

Potent and well-studied substances producing nonimmunologic immediate contact reactions (Table 60.4) include benzoic acid, sorbic acid, cinnamic acid, cinnamic aldehyde, and nicotinic acid esters. Under optimal conditions, more than half of the individuals react with local erythema and edema to these substances within 45 min of application if the concentration is high enough. Benzoic acid, sorbic acid, and sodium benzoate, preservatives for cosmetics and other topical preparations, are capable of producing immediate contact reactions at concentrations from 0.1 to 0.2% (Lahti, 1980; Soschin and Leyden, 1986).

Cinnamic aldehyde at a concentration of 0.01% may elicit erythema with a burning or stinging feeling in the skin. Some mouthwashes and chewing gums contain cinnamic aldehyde at concentrations that produce a pleasant tingling or "lively" sensation in the mouth and enhance the sale of the product. Higher concentrations produce lip swelling or contact urticaria.

The skin of the face, neck, back, and extensor sides of the upper extremities react more readily than other parts of the body; the soles and palms are the least sensitive areas (Gollhausen and Kligman, 1985; Lahti, 1980). Scratching does not enhance the reactivity, nor does occlusion, for benzoic acid.

The mechanism of NICU has not been established, but possible mechanisms are a direct influence upon dermal vessel walls or a nonantibody-mediated release of histamine, prostaglandins, leukotrienes, substance P, or other inflammatory mediators (Lahti and Maibach, 1987). No specific antibodies against the causative agent are in the serum.

It was earlier presumed that substances eliciting NICU also result in nonspecific histamine release from mast cells. However, antihistamines, hydroxyzine, and terfenadine did not inhibit reactions to benzoic acid, cinnamic acid, cinnamic aldehyde, methyl nicotinate, or dimethyl sulfoxide

TABLE 60.3
Agents Producing Immunologic Contact Urticaria (ICU)

- Animal Products
  - Amnion fluid
  - Blood
  - *Brucella aborrus* (Trunnell et al., 1985)
  - *Cercariae*
  - *Cheylerus malaccensis* (Yoshikawa, 1985)
  - Chironomidae, *Chironomus thummi thummi* (Mittelbach, 1983)
  - Cockroaches
  - Dander (Agrup and Sjostedt, 1985; Weissenbach et al., 1988)
  - *Dermestes macularus* Degeer (Lewis-Jones, 1985)
  - Gelatine (Wahl and Kleinhans, 1989)
  - Gut
  - Hair
  - *Listrophorus gibbus* (Burns, 1987)
  - Liver
  - Locust (Monk, 1988; Tee et al., 1988)
  - Mealworm, *Tenibrio molitor* (Bernstein et al., 1983)
  - Placenta
  - Saliva (Valsecchi and Cainelli, 1989)
  - Serum
  - Silk
  - Spider mite, *Terranychus urticae* (Reunala et al., 1983)
  - Wool
- Food
  - Dairy
    - Cheese
    - Egg
    - Milk (Boso and Brestel, 1987; Salo et al., 1986)
- Fruits
  - Apple (Halmepuro and Løwenstein, 1985; Pigatto et al., 1983)
  - Apricot
  - Banana
  - Kiwi
  - Mango
  - Orange
  - Peach
  - Plum
- Grains
  - Buckwheat (Valdivieso et al., 1989)
  - Maize
  - Malt
  - Rice (Lezaun et al., 1994)
  - Wheat
  - Wheat bran
- Honey
  - Nuts/seeds
  - Peanut
  - Sesame seed
  - Sunflower seed
- Meats
  - Beef
  - Chicken
  - Lamb
  - Liver
  - Turkey
- Seafood
  - Fish (Kavli and Moseng, 1987; Melino et al., 1987; Díaz Sánchez et al., 1994)
  - Prawns
  - Shrimp (Nagano et al., 1984)
- Vegetables
  - Beans
  - Cabbage
  - Carrot (Muñoz et al., 1985)
  - Celery (Kremser and Lindemayr, 1983; Wuthrich and Dietschi, 1985)
  - Chives
  - Cucumber
  - Endive
  - Lettuce
  - Onion
  - Parsley
  - Parsnip
  - Potato (Larkö et al., 1983)
  - Rutabaga (swede)
  - Tomato
  - Soybean
- Fragrances and flavorings
  - Balsam of Peru
  - Menthol
  - Vanillin
- Medicaments
  - Acetylsalicylic acid
  - Antibiotics
    - Ampicillin
    - Bacitracin
    - Cephalosporins
      - Cefotiam dihydrochloride (Mizutani et al., 1994)
    - Chloramphenicol (Schewach-Millet and Shpiro, 1985)
    - Gentamicin
    - Iodochlorhydroxyquin
    - Mezlocillin (Keller and Schwanitz, 1993)
    - Neomycin
    - Nifuroxime (Aaronson, 1969)
    - Penicillin (Rudzki and Rebandel, 1985)
    - Rifamycin (Grob et al., 1987)
    - Streptomycin
    - Virginiamycin (Baes, 1974)
  - Benzocaine (Kleinhans and Zwissler, 1980)
  - Benzoyl peroxide
  - Clobetasol 17-propionate (Gottmann-Lückerath, 1982)
  - Dinitrochlorobenzene (Valsecchi et al., 1986; van Hecke and Santosa, 1985)
  - Etophenamate (Pinol and Carapeto, 1984)
  - Fumaric acid derivatives (de Haan et al., 1994)
  - Mechlorethamine
  - Phenothiazines
    - Chlorpromazine (Lovell et al., 1986)
    - Levomepromazine (Johansson, 1988)
    - Promethazine
  - Pyrazolones
    - Aminophenazone (Lombardi et al., 1983)
    - Methamizole
    - Propylphenazone
  - TocopherolI (Kassen and Mitchell, 1974)

TABLE 60.3 (continued)
Agents Producing Immunologic Contact Urticaria (ICU)

Metals
- Copper (Shelley et al., 1983)
- Nickel (Valsecchi and Cainelli, 1987)
- Platinum
- Rhodium

Plant products (Lahti, 1986b)
- Algae
- Birch
- Camomile
- Castor bean
- Chrysanthemum (Tanaka et al., 1987)
- Cinchona (Dooms-Goossens et al., 1986a)
- Colophony (Rivers and Rycroft, 1987)
- Corn starch (Assalve et al., 1988; Fisher, 1987)
- Cotoneaster
- Emetin
- Fennel (La Rosa et al., 1986)
- Garlic
- *Grevillea juniperina* (Apted, 1988a)
- *Hakea suaveolens* (Apted, 1988b)
- Hawthorn, *Crataegus monogyna* (Steinman et al., 1984)
- Henna
- Latex rubber (Axelsson et al., 1987; Frosh et al., 1986; Morales et al., 1989; Spaner et al., 1989; van der Meeren and van Erp, 1986; Wrangsjo et al., 1988)
- Lichens
- Lily (Lahti, 1986a)
- Lime (Picardo et al., 1988)
- *Limonium tataricum* (Quirce et al., 1993)
- Mahogany
- Mustard (Kavli and Moseng, 1987)
- Papain (Santucci et al., 1985)
- Perfumes
- Pickles (Edwards and Edwards, 1984a)
- Rose (Kleinhans, 1985)
- Rouge
- Spices (Niinimaki, 1987)
- Strawberry (Grattan and Harman, 1985)
- Teak
- Tobacco (Tosti et al., 1987)
- Tulip (Lahti, 1986a)
- Winged bean (Lovell and Rycroft, 1984)

Preservatives and disinfectants
- Benzoic acid (Nethercott et al., 1984)

Benzyl alcohol
- Chlorhexidine (Bergqvist-Karlsson, 1988; Fisher, 1989; Nishioka et al., 1984)
- Chloramine
- Chiorocresol (Goncalo et al., 1987)
- 1,3-Diiodo-2-hydroxypropane (Löwenfeld, 1928)
- Formaldehyde (Andersen and Maibach, 1984; Lindskov, 1982)
- Gentian violet (Francois et al., 1970)
- Hexantriol (Tachibana et al., 1977)
- *para*-Hydroxybenzoic acid (Bottger et al., 1981)
- Parabens (Henry et al., 1979)
- Phenylmercuric propionate
- *ortho*-Phenylphenate (Tuer et al., 1986)
- Polysorbates
- Sodium hypochlorite

Sorbitan monolaurate

Tropicamide (Guill et al., 1979)

Enzymes
- *alpha*-Amylasc (Morren et al., 1993)
- Cellulases (Tarvainen et al., 1991)
- Xylanases (Tarvainen et al., 1991)

Miscellaneous
- Acetyl acetone (Sterry and Schmoll, 1985)
- Acrylic monomer
- Alcohols (amyl, butyl, ethyl, isopropyl) (Rilliet et al., 1980)
- Aliphatic polyamide
- Ammonia
- Ammonium persulfate
- Aminothiazole
- Benzophenone
- Butylated hydroxytoluene
- Carbonless copy paper
- Chlorothanil (Dannaker et al., 1993)
- Cu(II) acetyl acetonate (Sterry and Schmoll, 1985)
- Denatonium benzoate
- Diethyltoluamide
- Epoxy resin (Jolanki et al., 1987)
- Formaldehyde resin
- Lanolin alcohols
- Lindane
- Methyl ethyl ketone (Varigos and Nurse, 1986)
- Monoamvlamine
- Naphtha (Goodfield and Saihan, 1988)
- Naphihylacetic acid (Camarasa, 1986)
- Nylon (Dooms-Goossens et al., 1986b; Hatch and Maibach, 1985)
- Oleylamide
- Paraphenylenediamine (Edwards and Edwards, 1984b; Temesvari, 1984)
- Patent blue dye
- Perlon
- Phosphorus sesquisulfide (Payero et al., 1985)
- Plastic
- Polypropylene (Tosti et al., 1986)
- Polyethylene glycol
- Potassium ferricyanide
- Seminal fluid (Blair and Parish, 1985)
- Sodium silicate
- Sodium sulfide
- Sulfur dioxide
- Terpinyl acetate
- Textile finish (de Groot and Gerkens, 1989)
- Vinyl pyridine
- Zinc diethyldithiocarbamate

but they did inhibit reactions to histamine in the prick test (Lahti, 1980, 1987). The results suggest that histamine is not the main mediator in NICU.

60.3.3.1 Effect of Nonsteroidal Anti-Inflammatory Drugs

Nonimmunologic contact reactions to benzoic acid, cinnamic acid, cinnamic aldehyde, methyl nicotinate, and diethyl fumarate can be inhibited by peroral acetylsalicylic acid and

TABLE 60.4
Agents Producing Nonimmunologic Contact Urticaria (NICU)

- Animals
  - Arthropods
  - Caterpillars (Ducombs et al., 1983; Edwards et al., 1986)
  - Corals
  - Jellyfish
  - Moths
  - Sea anemones
- Foods
  - Cayenne pepper
  - Cow's milk (Oranje et al., 1992, 1994)
  - Fish
  - Mustard
  - Thyme
- Fragrances and flavorings
  - Balsam of Peru
  - Benzaldehyde
  - Cassia (cinnamon oil)
  - Cinnamic acid
- Cinnamic aldehyde (Emmons and Marks, 1985; Guin et al., 1984; Larsen, 1985; Helton and Storrs, 1994)
- Medicaments
  - Alcohols (Wilkin and Fortner, 1985)
  - Benzocaine
  - Camphor
  - Cantharides
  - Capsaicin
  - Chlorophorm
  - Dimethyl sulfoxide
  - Friar's balsam
  - Iodine
  - Methyl salicylate
  - Methylene green
  - Myrrh
  - Nicotinic acid esters
  - Resorcinol
  - Tar extracts
  - Tincture of benzoin
  - Witch hazel
- Metals
  - Cobalt
- Plants
  - Nettles (Kulze and Greaves, 1988; Oliver et al., 1991)
  - Seaweed
- Preservatives and disinfectants
  - Acetic acid (Burrall et al., 1990)
  - Benzoid acid
  - Chlorocresol (Freitas and Brandão, 1986)
  - Formaldehyde
  - Sodium benzoate
  - Sorbic acid (Soschin and Leyden, 1986)
- Miscellaneous
  - Butyric acid
  - Diethyl fumarate (Lahti and Maibach, 1985a; White and Cronin, 1984)
  - Histamine
  - Pine oil
  - Propylene glycol (Funk and Maibach, 1994)
  - Pyridine carboxaldehyde (Archer and Cronin, 1986; Hannuksela et al., 1989)
  - Sulfur (Böttger et al., 1981)
  - Turpentine

indomethacin (Lahti et al., 1983, 1987) and by topical application of diclofenac or naproxene gels (Johansson and Lahti, 1988). Inhibition by acetylsalicylic acid can last up to 4 days (Kujala and Lahri, 1989). The mechanism by which nonsteroidal anti-inflammatory drugs (NSAIDs) inhibit contact reactions in human skin has not been defined, but it may be ascribed to a common pharmacological action, that is, inhibition of prostaglandin bioformation.

60.3.3.2 Role of Sensory Nerves

Capsaicin (*trans*-8-methyl-*N*-vanillyl-6-nonenamide), the most abundant of the pungent principles of the red pepper (*Capsicum*), is known to induce vasodilatation and protein extravasation by specific release of bioactive peptides, for example, substance P, from axons of unmyelinated C-fibers of the sensory nerves. Pretreatment with capsaicin inhibits erythema reactions in histamine skin tests (Bernstein et al., 1981; Walleneren and Moller, 1986). However, pretreatment with capsaicin inhibited neither erythema nor edema elicited by benzoic acid or methyl nicotinate (Larmi et al., 1989). The result suggests that pathways sensitive to capsaicin are not substantially involved.

Topical anesthesia (lidocaine plus prilocaine) can inhibit erythema and edema reactions to histamine and also to benzoic acid and methyl nicotinate, but it is not known whether the inhibitory effect is due to the influence on the sensory nerves of the skin only, or if the anesthetic affects other cell types or regulatory mechanisms of immediate-type skin inflammation (Larmi et al., 1989).

60.3.3.3 Effect of Ultraviolet Irradiation

Immediate contact reactions to benzoic acid and methyl nicotinate can also be inhibited by ultraviolet (UV) B and A light exposure, an effect that lasts for at least 2 weeks (Larmi et al., 1988). An interesting observation was the fact that UV irradiation had systemic effects; it inhibited reactions on nonirradiated skin sites too (Larmi, 1989). The mechanism of UV inhibition is not known, but it does not seem to be due to thickening of the stratum corneum (Larmi, 1989).

Little is known about the histology of immediate contact reactions. In studies with nicotinates, the accumulation of mononuclear cell perivascular infiltrate was seen from 15 min and that of neutrophils from 2 h onward, persisting up to 48 h in normal subjects. Leukocytoclasis was also observed. The cell infiltrate was seen to a lesser degree in one of six atopic eczema patients but not in normal subjects treated with 600 mg acetylsalicylic acid before the nicotinate application (Daroczy and Temesvari, 1988; English et al., 1987).

60.3.3.4 Animal Model for Nonimmunologic Contact Urticarial Reactions

Animal models permit identification of agents capable of immediate contact reactions and mechanistic studies (Lahti, 1988). Guinea pig body skin reacts with rapidly appearing erythema to cinnamic aldehyde, methyl nicotinate, and

dimethyl sulfoxide but not to benzoic acid, sorbic acid, or cinnamic acid. Any of these substances applied to the guinea pig earlobe causes erythema and edema to appear. Quantification of edema by measuring changes in the ear thickness with a micrometer caliper is an accurate, reproducible, and rapid method (Lahti and Maibach, 1984).

Analogous reactions can be elicited in the earlobes of other laboratory animals. Cinnamic aldehyde and dimethyl sulfoxide produce ear swelling in the rat and mouse, but benzoic acid, sorbic acid, cinnamic acid, diethyl fumarate, and methyl nicotinate produce no response. This suggests that either several mechanisms are involved in immediate contact reactions from different substances or there are differences in the activation of mediators of inflammation between guinea pig, rat, and mouse (Lahti and Maibach, 1985a, 1985b).

The swelling response in the guinea pig earlobe is dependent on the concentrations of the eliciting substance. The maximal response is a roughly 100% increase in ear thickness, which appears within 50 mm of application.

Biopsies taken from the guinea pig earlobe 40 mm after application of test substances show marked dermal edema and intra- and perivascular infiltrates of heterophilic (neutrophilic in humans) granulocytes: They appear to be the characteristic of NICU in the guinea pig ear (Anderson, 1988; Lahti and Maibach, 1984; Lahti et al., 1986).

A decrease in response to contact urticaria is noticed after reapplication of the test substances to the guinea pig ear on the following day (Lahti and Maibach, 1985c). The tachyphylaxis is not specific to the substance that produces it, and the reactivity to other agents decreases as well. The length of the refractory period varies with the compound used. It is 4 days for methyl nicotinate, 8 days for diethyl fumarate and cinnamic aldehyde, and up to 16 days for benzoic acid, cinnamic acid, and dimethyl sulfoxide.

The reaction of guinea pig earlobe to NICU seems to be similar to that of human skin. The similarities include the morphology, the time course of maximal response, the concentrations of the eliciting substances, the tachyphylaxis phenomenon (Lahti, 1980), and the lack of an inhibitory effect of antihistamines on contact reactions (Lahti, 1987; Lahti et al., 1986).

60.3.3.5 Specificity of the Reaction

Pyridine carboxaldehyde (PCA) is one of the many substances that can produce nonimmunologic immediate contact reactions (Archer and Cronin, 1986). It has three isomers: 2-, 3-, and 4-PCA, according to the position of the aldehyde group on the pyridine ring. 3-PCA is the strongest and 2-PCA the weakest contact reactant in both the human skin and guinea pig ear swelling test (Hannuksela et al., 1989; Lahti and Maibach, 1984). Only a slight change in the molecular structure of a chemical can greatly alter its capacity to produce nonimmunologic immediate contact reactions.

60.4 DIAGNOSTIC TESTS

The diagnosis of immediate contact reactions is based on a full medical history and on skin tests with suspected substances.

60.4.1 Tests for Both Immunologic and Nonimmunologic Contact Urticaria

The simplest test is the open test. For this test, the suspected substance (fish, apple, and carrot) is applied and gently rubbed on either normal-looking or slightly affected skin, usually the hand. The test site is observed for 60 min (Hannuksela, 1986). A positive result is seen as an edema and erythema reaction or as tiny intraepidermal spongiotic vesicles typical of acute eczema.

The use test requires the patient to handle the suspected agent precisely as handled when symptoms appeared. Wearing surgical gloves on wet hands to provoke contact urticaria to latex is a typical use test.

In the open test, 0.1 mL of the test substance is spread on a 3×3 cm area of the skin of the upper back or on the extensor side of the upper arm. The test should first be performed on nondiseased skin and then, if negative, on previously or currently affected skin (Lahti and Maibach, 1986). Even in immunologic contact urticaria there may be a marked difference between skin sites in their capacity to elicit contact urticaria (Maibach, 1986). This is typical of NICU. The face has been considered the most sensitive skin area (Gollhausen and Kligman, 1985). Often it is desirable to apply contact urticants to skin sites suggested by the patient's history. The immunologic contact reactions usually appear within 15–20 min and nonimmunologic ones within 45–60 min after application. A positive reaction comprises a wheal-and-flare reaction and sometimes a vesicular eruption indistinguishable from that seen in eczema (Hjorth and Roed-Petersen, 1976).

60.4.2 Tests for Immunologic Contact Urticaria

Open application as already described is all that is required for most ICU agents. Prick testing is often the method of choice for testing patients with suspected allergic contact reactions when the open application method is negative.

The scratch test is a less-standardized method than the prick test, but it is useful when nonstandardized allergens must be used (Paul, 1987). The allergen solutions in scratch testing are the same as those used in prick testing. Also, freeze-dried and other powdered allergens moistened with 0.1 N aqueous sodium hydroxide solution and fresh foods (e.g., potato, apple, and carrot) can be used. When testing with poorly standardized or nonstandardized substances, control tests should be made on at least 20 people to avoid false interpretation of the test results.

The chamber scratch test was introduced for testing foods when commercial allergens with proven efficacy are not available (Hannuksela and Lahti, 1977). Potato, apple, and carrot lose their allergenicity when cooked, deep-frozen, or made into juice, and it is therefore best to use them fresh for skin testing.

In the chamber scratch test, the procedure is that of the ordinary scratch test but the scratch and the foodstuff are covered with a small aluminum chamber (Finn Chamber,

Epitest Ltd Oy, Hyryla, Finland) for 15 min. The result is read 5 min after the removal of the chamber according to the criteria of the scratch test. Reactions at least the size of a similarly produced histamine reaction are usually clinically significant. Histamine hydrochloride (10 mg/mL) is the positive reference and aqueous 0.1 N sodium hydroxide the negative reference.

The Prausnitz–Kustner test or passive-transfer test has been used in occupational dermatology for detecting immunologic contact urticaria to potato (Tuft and Blumstein, 1942) and to rubber (Kopman and Hannuksela, 1983). Today, this would generally be limited to animal studies.

RAST is seldom needed for contact urticaria diagnosis, but RAST inhibition tests are used in investigating cross-allergenicity (Halmepuro and Løvenstein, 1985). For this purpose, crossed radioimmunoelectrophoresis and its inhibition are also used.

NSAIDs and antihistamines should not be taken by patients during tests for immediate contact reactions because these drugs may inhibit the reactions. Using the same test site repeatedly may result in the tachyphylaxis phenomenon and cause false negative results.

A positive open test (erythema alone or wheal and flare) is almost always of clinical significance in ICU, assuming that the same agent is not reactive in controls. With skin prick and scratch testing, great caution must be utilized to rule out nonspecific reactions.

Caution: In testing for ICU when other organs are also involved, very small doses and dilute solutions are indicated to avoid reproducing systemic reactions (von Krogh and Maibach, 1982). Facilities for resuscitation should also be available.

When examining patients with a suspected allergic contact reaction, the prick, scratch, or scratch-chamber tests may be done first because the test procedures are fast. The diagnosis should be based on the result of the open application test and interpreted by reviewing the clinical history and the background controls.

60.5 SUMMARY

In clinical practice, patients report immediate contact reactions after applying cosmetics or therapeutic agents and after handling food products. Not only have dermatologists and allergists been uncertain about the nature of these reactions, but manufacturers, their toxicologists, and other involved personnel have had difficulty in understanding this type of reaction and in developing less irritating products. Studies on the mechanisms of immediate contact reactions from different substances and the standardization of human and animal tests for these reactions are a challenge for future research. Dermatologists, allergists, toxicologists, and medical authorities need to combine their efforts to investigate the capacity of various environmental agents to produce immediate contact reactions, as was done in the past for delayed-type skin effects.

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61 *In Vitro* Approaches to Assessment of Skin Irritation and Phototoxicity of Topically Applied Materials

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61.1 SKIN IRRITATION

61.1.1 Introduction

Skin irritation is a deceptively simple phenomenon. The reality however is very different: skin irritation responses range from a variety of sensory effects, through minor degrees of acute reaction, characterized by erythema, or of cumulative irritancy, characterized by erythema and dryness to more profound degrees of response, including burning/corrosion with consequent scar formation. Such matters are fully covered elsewhere (e.g., Chew and Maibach, 2006, as well as in this book), but for the purpose of this chapter, the focus must be on the evaluation of the acute irritant response. This is simply a reflection of the historic use of the rabbit Draize test (Draize et al., 1944) as the means of identifying those substances that present a skin irritation hazard (EC, 1992; EC, 1998). In this test, a 4-h semi-occlusive treatment with undiluted material, followed by assessments of erythema and edema up to 72 h in three rabbits, has been deployed to discriminate between substances in the following categories:

- Causes severe burns
- Causes burns
- Irritant
- Mild irritant
- Not classified

These categories are rather arbitrary; "not classified" means simply not irritant enough to present an acute hazard. In many areas, including in the European Union, there is no separation between the irritant and mild irritant category. The relevance of these classifications to real human hazard has been questioned for many years (Phillips et al., 1972; Nixon et al., 1975; Robinson et al., 1998). They have almost no place at all in the assessment of the risk to human health except for the materials which can cause burns (Basketter et al., 2006), where the obvious advice is to avoid any direct skin contact with the neat material. However, for irritant materials, cumulative effects of the formulation rather than the response to a single exposure of an isolated chemicals is overwhelmingly the issue (Hannuksela and Hannuksela, 1995; Hall-Manning et al., 1995).

Set against the above background, there have been many efforts to use *in vitro* models to predict skin irritation and these have formed the subject of relatively recent detailed review (Welss et al., 2004; Botham, 2004). However, it is worthwhile to note that there is an important division between *in vitro* approaches to the identification of corrosive materials

and methods which aim to separate irritant substances from those with little effect on the skin. The identification of corrosive substances has proven a relatively simple task, with an optimal strategy falling out readily from the handful of methods (some officially regarded as validated), which have been proposed to address this end point (OECD 2002, 2002a & 2002b). This contrasts with the position for the differentiation of irritant/mild irritant/nonirritant discrimination. It is not appropriate here to undertake a detailed/historical review; instead, the main points are highlighted and illustrated.

In the past, skin irritation was assumed to be a fairly simple response to direct toxic insult and thus likely to be readily predicted by observations of the response of cells *in vitro* to chemical exposure, particularly by measurements of cytotoxicity, for example the release of the cytoplasmic enzyme lactate dehydrogenase (Harvell et al., 1992) or the release of mediators such as arachidonic acid (DeLeo et al., 1996). Although the cells deployed for this purpose were varied and historically dermal fibroblasts were easy to culture, keratinocytes usually were favored as the main cell of the epidermis (Gajjar and Benford, 1987/88; Chamberlain and Earl, 1994; Cohen et al., 1994). In more recent years, the focus has moved very largely to the deployment of 3-D skin models, largely based on the argument that these provide a skin barrier (of sorts) and will thus help to take into account the excessive bioavailability of materials applied directly to monolayer culture, this being one reason proffered for the failure of simple keratinocyte systems to predict skin irritation hazard with any real accuracy (Roguet et al., 1994; Cotovio et al., 2005; Kandarova et al., 2005; Tornier et al., 2006).

61.1.2 Validation Status of *In Vitro* Assays for Corrosion and Irritation

61.1.2.1 Corrosion

Three methods for assessing skin corrosion have been validated and accepted into international regulations. These are the rat skin transcutaneous electrical resistance (TER) test, human skin model tests such as EpiSkin™ and EpiDerm™, and the Corrositex™ test. Prevalidation of these assays was carried out under the auspices of ECVAM and reported as the outcome of ECVAM workshop 6 (Botham et al., 1995). In this study, the rat skin TER, a human skin model (Skin^{2}™), and Corrositex were tested in a blind trial using 25 corrosives and 25 noncorrosives by two or three laboratories. The recommendations made at this workshop led to carrying out of a formal validation study of these methods (Barratt et al., 1998; Fentem et al., 1998). In the validation trial, 60 coded chemicals were tested by three laboratories in each assay and in addition to Skin^{2}, a second human skin model was also included in the study. During the trial, however, the Skin^{2} skin model ceased commercial production, despite the EpiSkin method proving successful. Further validation studies were subsequently carried out using another commercially available model EpiDerm (Liebsch et al., 2000).

The rat skin TER test uses excised rat skin as a test system and its electrical resistance as an end point. Test materials are applied for up to 24 h to the epidermal surfaces of skin discs. Corrosive materials are identified by their ability to produce a loss of normal stratum corneum integrity and barrier function, which is measured as a reduction in the TER below a threshold level (Oliver et al., 1986). For rat TER, a cut-off value of 5 kΩ has been selected. Generally, materials that are noncorrosive in animals but are irritating or nonirritating do not reduce the TER below this cut-off value. A dye-binding step is incorporated into the test procedure for confirmation testing of positive results in the TER including values around 5 kΩ. The dye-binding step determines if the increase in ionic permeability is due to physical destruction of the stratum corneum. The rat skin TER method has been shown to be predictive of corrosivity in the *in vivo* rabbit test (OECD Test Guideline 404; OECD, 2002a). The TER method can also be applied to excised human skin and some differences between human and rat skin have been reported (Whittle and Basketter, 1994). The rat skin TER was included by the EU in Annex V of the Dangerous Substances Directive (EU, 2000) and an OECD guideline has been published (OECD Test Guideline 430; OECD, 2002).

Human skin model tests use cell viability as an end point measured, for example, by the reduction of MTT (Mossman, 1983). The principle of the human skin model assay is based on the hypothesis that corrosive chemicals are able to penetrate the stratum corneum by diffusion or erosion, and are cytotoxic to the underlying cell layers. Test materials are applied to the surface of the human skin model and corrosive materials are identified by their ability to produce a decrease in cell viability below defined threshold levels at specified exposure periods. The human skin model method was included by the EU in Annex V (EU, 2000) and an OECD guideline has been published (OECD Test Guideline 431; OECD, 2002b). The OECD guideline provides guidance on the general and functional properties required for a skin model to be suitable for use in the test and on reference chemicals suitable for testing the predictive ability of the model. Following the original validation of the EpiSkin and EpiDerm models and publication of the guideline, data for two other human models (SkinEthic™ and CellSystems® EST1000) have been published showing their applicability (Kandarova et al., 2006; Hoffmann et al., 2005).

The Corrositex test employs penetration of test substances through a hydrogenated collagen matrix (biobarrier) and supporting filter membrane. Following the validation study and review of available data by the ECVAM scientific advisory committee (ESAC) it was concluded that the Corrositex test was a scientifically validated test, but only for those acids, bases, and their derivatives which met the technical requirements of the assay (NIH, 1999; ECVAM, 2001). Corrositex was not adopted into Annex V at the same time as the rat skin TER and human skin model tests but it has been accepted by the US Department of Transport (US DOT) and a draft test guideline (DTG 435 *In Vitro*

Barrier Membrane Test), based on the Corrositex test method, is under consideration by the OECD.

61.1.2.2 Irritation

There are no validated *in vitro* assays for skin irritation; however, an ECVAM sponsored validation study of *in vitro* methods for acute skin irritation is currently running, the outcome of which was expected by the end of 2006 (Fentem and Botham, 2002) and was finally approved in April 2007. Prior to this, validation study work was started in 1999 on prevalidation of the then most promising tests (Botham et al., 1998). These were two human 3-D skin model cytotoxicity protocols using EpiDerm and EpiSkin (MTT reduction end point), Prediskin™ (measuring cytotoxicity to *ex vivo* human skin cultures), the nonperfused pig ear model (measuring transepidermal water loss (TEWL) from the skin surface), and the *in vitro* mouse skin integrity function test (SIFT) which used TEWL and electrical resistance as the end points. The conclusion from this prevalidation study was that none of the tests were ready for progression to formal validation (Fentem et al., 2001). Various follow-up activities then took place, which enabled some of the test protocols to meet the criteria for inclusion in a formal validation study (Zuang et al., 2002). The protocols for the two human 3-D skin models, EpiDerm and EpiSkin, were harmonized to allow a common approach for skin models to be investigated (Portes et al., 2002; Cotovio et al., 2005; Kandarova et al., 2004, 2005). This approach uses a short application time (15 min) followed by a recovery incubation of 42 h before cytotoxicity measurement. Chemicals are predicted as irritant if they reduce mean culture viability to $<$50% of untreated controls. The SIFT data analysis and prediction model were also modified to enable it to enter formal validation (Heylings et al., 2003).

The current validation study is aimed at the discrimination of those materials which would be classified as irritants (R36) from nonirritants (no classification), which is the basis on which the test chemicals have been selected. However, there will also be a retrospective assessment of the data with respect to prediction of the three globally harmonised system (GHS) categories (irritant/mild irritant/no classification; OECD, 2001). Phase 1 of the validation study involved testing a set of 20 chemicals under blind conditions with the three assays in the respective lead laboratories only (Liebsch et al., 2005). The performance of the two 3-D skin model tests was sufficiently promising for them to progress to phase 2, but the predictive ability of the SIFT was not considered sufficient for it to progress to the next phase. Phase 2 experimental testing of 60 coded chemicals by six laboratories (three per skin model) took place from October 2004 to April 2005. In addition to cytotoxicity (measured by MTT reduction), a second end point, release of the cytokine IL1-α, has also been explored for the EpiSkin model as a secondary predictor of irritants for those chemicals predicted as nonirritant by the MTT assay. Since then independent statistical analysis and management team review of the data has taken place and the positive review of the trial by ESAC occulled in April 2007. Since this validation trial was successful it is likely that other available skin models will undergo performance testing for their suitability for the same approach.

61.1.3 Safety Assessment of Substances and Preparations

The assessment of safety can be considered in two separate parts: the risk to health from exposure to an individual substance, which largely relates to occupational health, and the risk to consumer and occupational health from exposure to preparations, e.g., personal care products, medicaments, and cleaning products. In the former case, occupational dermatitis is known to arise from exposure to single substances, although typically this is accidental exposure to corrosive materials (Basketter et al., 2006), whereas occupational acute irritation is relatively rare. Much more common is cumulative irritancy driven by repeated exposure to preparations, typically in association with a substantial degree of wet work. Assessment of the ability of products to cause skin irritation in response to repeated exposure forms the subject of a number of reviews (Robinson and Perkins, 2002; Cooper et al., 2005), and typically involves clinical studies, which will not be repeated here. However, Figure 61.1 presents in generic form the type of assessment that may be considered using *in vitro* models. Some simple rules are necessary. The *in vitro* test must be one for which there is considerable experience in the institution; the substances/products to be tested must be of similar type; there must be knowledge that the relative irritating ability of these substances/products clinically is reflected by the results from the *in vitro* test. In such a situation, it may be relatively straightforward to simply compare the dose–response curve for a known material with an unknown substance/product. The dose–response displayed in Figure 61.1 displays an increasing response (the values are arbitrary), which suddenly falls as cell death dominates at the highest concentration. However, it can be seen that the test material behaves similarly to the known control, albeit

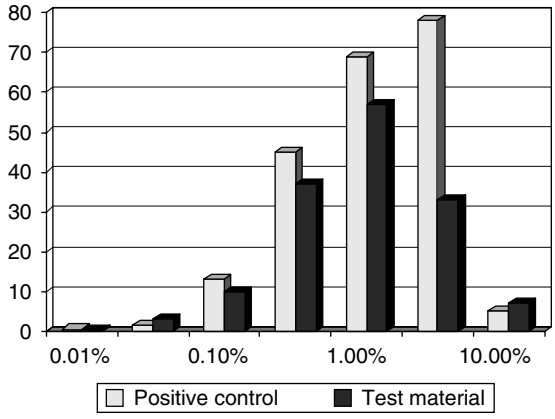


FIGURE 61.1 Evaluation of relative irritancy using a simple *in vitro* procedure.

giving a slightly lower degree of response. In this situation, one might readily conclude that the test material, whether a substance or a product, is likely to be just a little less irritating to the skin. Such a conclusion is entirely dependent on the quality of the historic evaluation of the *in vitro* assay against clinical data.

61.1.4 Future Prospects

As mentioned earlier, evaluation of the end point of skin irritation is important for human health. However, the current *in vitro* strategies very largely focus on the classification of intrinsic hazards of chemicals, rather than permitting the risk to human health to be assessed. Most important in this respect is the assessment of the propensity of a formulation to cause skin irritation as a consequence of repeated exposure. Only very limited efforts to model this *in vitro* have been reported (de Brugerolle de Fraisinette et al., 1999). Until the *in vitro* methods for simple hazard identification/classification have been formally validated and gained a reasonable degree of acceptance, it seems unlikely that great progress will be made in the more directly health-related development and deployment of more sophisticated *in vitro* tests for skin irritation.

61.2 *IN VITRO* PHOTOTOXICITY TESTING—BACKGROUND

Safety tests for phototoxicity historically have used animal models (reviewed by Lambert et al., 1996), largely involving guinea pigs. However, there has been no standardization of procedures and it can be difficult to compare results from different laboratories (Maurer, 1987; Spielmann et al., 1994a). Draft proposals for OECD guidelines for phototoxicity testing *in vivo* were considered in 1991 and 1995 (OECD, 1991, 1995). However, these proposals were never progressed largely because of the unwillingness to define further animal models and because of pressure from consumers and legislators, notably in Europe, to introduce *in vitro* alternative models. In addition, developments in cell culture and bioanalytical techniques stimulated renewed interest in the use of *in vitro* models for safety hazard evaluation (Balls et al., 1990). A task force on phototoxicity testing *in vitro* was set up on the initiative of the European Cosmetic Toiletry and Perfumery Association (COLIPA), which was joined in a joint project by the European Union (EU) through the European Centre for the Validation of Alternative Methods (ECVAM). *In vitro* phototoxicity testing was the topic of the second ECVAM workshop that was organized in 1993 in collaboration with the COLIPA task force on phototoxicity testing (Spielmann et al., 1994a). The aim of this workshop was to plan a validation study on the most promising *in vitro* phototoxicity tests and to identify an optimum set of test chemicals, based on high quality *in vivo* data in humans. A list of high quality data from standardized human photopatch testing for both acute phototoxicity and photoallergy was made available. A total of three studies were subsequently conducted to validate the 3T3 neutral red uptake phototoxicity test (NRU PT), which has now been accepted for regulatory purposes within the European Union and by the OECD (see section 61.2.1). A second ECVAM workshop on *in vitro* phototoxicity testing was held in 1999. The status of testing methods and their development since the first ECVAM workshop was discussed and a report published, focusing on human photopatch testing, *in vitro* photogenotoxicity, *in vitro* methods for assessing the photoallergy potential of chemicals, the application of 3-D human skin models for *in vitro* phototoxicity testing, and general testing strategies covering all aspects of *in vitro* phototoxicity (Spielmann et al., 2000).

61.2.1 Validation Status of *In Vitro* Phototoxicity Assays

61.2.1.1 Photoirritation

An *in vitro* photocytotoxicity model using mouse 3T3 fibroblasts with neutral red uptake as end point (the 3T3 NRU PT) was first developed and prevalidation started in 1992. This test is based on a comparison of the cytotoxicity of a chemical when tested in the presence and in the absence of exposure to a noncytotoxic dose of simulated solar light (UVA/visible spectrum). Cytotoxicity is expressed as a concentration-dependent reduction of the uptake of the vital dye neutral red when measured 24 h after treatment with the test chemical. Substances identified by this test are likely to be phototoxic following systemic application and distribution to the skin, or after topical application. Prevalidation was carried out by eight laboratories in a nonblind trial using 20 chemicals (11 phototoxic and 9 nonphototoxic) and a prediction model using a photoirritation factor was developed (PIF: EC_{50} value $-$UV/EC_{50} value $+$UV) to discriminate between positive and negative chemicals (Spielmann et al., 1994b). Using a cut-off value of PIF = 5, all of the test chemicals were correctly classified in the 3T3 NRU PT. Similar results were obtained in an independent study conducted at the Hatano Research Institute, in Japan, in 1994, using the same test protocol and the same chemicals (Wakuri et al., 1995). The following formal blind validation trial using 30 test chemicals (25 phototoxic and 5 nonphototoxic) demonstrated that the test was reproducible in the nine participating laboratories, and that correlation between *in vitro* and *in vivo* phototoxic potential was very high, with all phototoxic chemicals being correctly identified (Spielmann et al., 1998a). At the request of the Scientific Committee of Cosmetology and Non-Food Products (SCCNFP), the expert advisory committee on cosmetics, a set of the most commonly used UV-filter chemicals, which are not phototoxic *in vivo* and poorly soluble in water plus a set of known phototoxic chemicals, were tested in a further blind trial with the 3T3 NRU PT (20 chemicals in four laboratories). The test was found to correctly assess the phototoxic potential of modern UV-filter chemicals (Spielmann et al., 1998b). The NRU PT protocol can also be used with human keratinocytes, as demonstrated in a blind study with the chemicals of the EU/COLIPA validation study and the

UV-filter study (Clothier et al., 1999). The 3T3 NRU PT, because of its successful validation, has been officially accepted by the European Commission and the EU Member States into Annex V of EU Council Directive 67/548/EEC for the classification and labeling of hazardous chemicals (Anon., 2000). An OECD guideline for the NRU PT (OECD Test Guideline 432) was accepted and published in 2004 (OECD, 2004). For TG432, a modified prediction model was developed based on the results of the validation trials; a test substance with a PIF < 2 predicts: "no phototoxicity," a PIF >2 and <5 predicts: "probable phototoxicity," and a PIF > 5 predicts: "phototoxicity." TG432 also reflects the conclusions of the validation trials that false positive results may be obtained at high test concentrations and recommends a maximum test concentration of 1000 μg/mL.

Although the NRU PT is the only validated *in vitro* test for photoirritation, additional tests also exist. A photo-red blood cell hemolysis test (Photo-RBC) has undergone prevalidation evaluation using the 30 chemicals used in the NRU PT validation (Pape et al., 2001). In the protocol for the Photo-RBC test, two end points are determined in erythrocytes, namely, photohemolysis as a measure of primary type II photoreactions and methemoglobin formation (met-Hb) as a measure of type I photoreactions. There were some problems with interlaboratory transferability of the protocol, but the overall conclusion of the prevalidation study was that the Photo-RBC test can be performed reproducibly and it provides relevant mechanistic information on photoreactions for use within a wider testing strategy. RBCs are also resistant to UVB, which enables exposure to the entire solar spectrum, compared to the NRU PT. Yeast (*Saccharomyces cerevisiae*) is also relatively insensitive to both sunlight and prolonged exposure to test materials which has also led to its proposal for use in both phototoxicity and photogenotoxicity tests (using mutant strains deficient in DNA repair pathways). These tests have not undergone any validation activity but were reviewed by the second ECVAM phototoxicity workshop (Spielmann et al., 2000).

The NRU PT has been demonstrated to detect the phototoxic potential of both strong and weak phototoxins irrespective of their aqueous solubility (Spielmann et al., 1998b). In the case of negative responses, however, there may be some uncertainty. This arises where the chemical can be tested only at low concentration because of lack of aqueous solubility. Assays using 3-D reconstructed human skin models can help address this. 3-D skin models allow the application of various types of test materials (undiluted and using both aqueous and organic solvents) and preparations to their surface and, therefore, have fewer solubility problems. 3-D skin models are considerably less sensitive to UVB than monolayer cells (Cohen et al., 1994; Corsini et al., 1997). This allows the possibility of using a light source emitting, in addition to UVA, a greater proportion of UVB compared with sources used for monolayer cell models and thus further mimicking sunlight (Jones et al., 2001). Skin^2 (a now unavailable commercial model) was originally reported to identify phototoxic hazard potential (Edwards, 1994; Liebsch, 1995; Api, 1997) in a manner similar to the NRU PT with evaluation of phototoxic hazard potential via an MTT viability assay (Mossman, 1983). Similar protocols have since been successfully transferred to currently available 3-D skin models such as EpiDerm, EpiSkin, and SkinEthic (Cohen et al., 1994; Roguet et al., 1994; Augustin et al., 1997; Liebsch et al., 1997; Bernard et al., 1999; Jones et al., 1999; Medina et al., 2001; Jones et al., 2001). EpiDerm has also undergone evaluation in an ECVAM prevalidation study, which gave good predictions in three laboratories using 10 chemicals (Liebsch et al., 1999). Being relatively expensive, these models are not suitable to use in a screen to predict phototoxic hazard but are useful in the further evaluation of materials for topical application in a testing strategy.

61.2.1.2 Photoallergy

The results of the validation trial of the NRU PT showed that the model detected both photoirritant and photoallergic chemicals; however, there are no validated *in vitro* tests for photoallergy *per se*. Photoallergy is a delayed-type hypersensitivity with an essential requirement for UV radiation (Stephens and Bergstresser, 1985). Photochemical binding of photoallergens to protein is widely accepted as the initial step of the photoallergenic process and has been proposed as a test for potential photoallergenicity (Barratt and Brown, 1985; Pendlington and Barratt, 1990). Photoirritants may also photobind to protein, but in this case other photochemical reactions would be expected to be more significant and photooxidation of histidine has been proposed to identify photooxidising potential which may lead to photoirritancy (Lovell, 1993). Efficient photooxidizers may be considered photoirritant rather than photoallergic. A photobinding assay using binding to human serum albumin, in conjunction with a test of photooxidation of histidine, was used to test the 30 chemicals used in the NRU PT validation trial (Lovell and Jones, 2000). Six of seven photoallergens were identified as such by the photobinding assay. Most photoirritants also caused photomodification of protein, but 11 (out of 17) also photooxidized histidine efficiently and so were classified as photoirritants. Four photoirritants remained falsely predicted as photoallergens. Two photoirritants were negative for both photomodification of protein and histidine photooxidation. Four chemicals negative *in vivo* were negative *in vitro*. The remaining two chemicals could not be classified because of unclear data both *in vivo* and *in vitro*. There was, therefore, good detection of photoallergens. Differentiation between photoallergens and phototoxins was seen but not achieved in all cases.

61.2.2 Safety Assessment of Substances and Preparations

Where substances are intended for use in products either intended for skin application or which may come into contact with the skin, it is necessary to carry out an assessment of potential phototoxic hazard. Figure 61.2 gives a step-wise

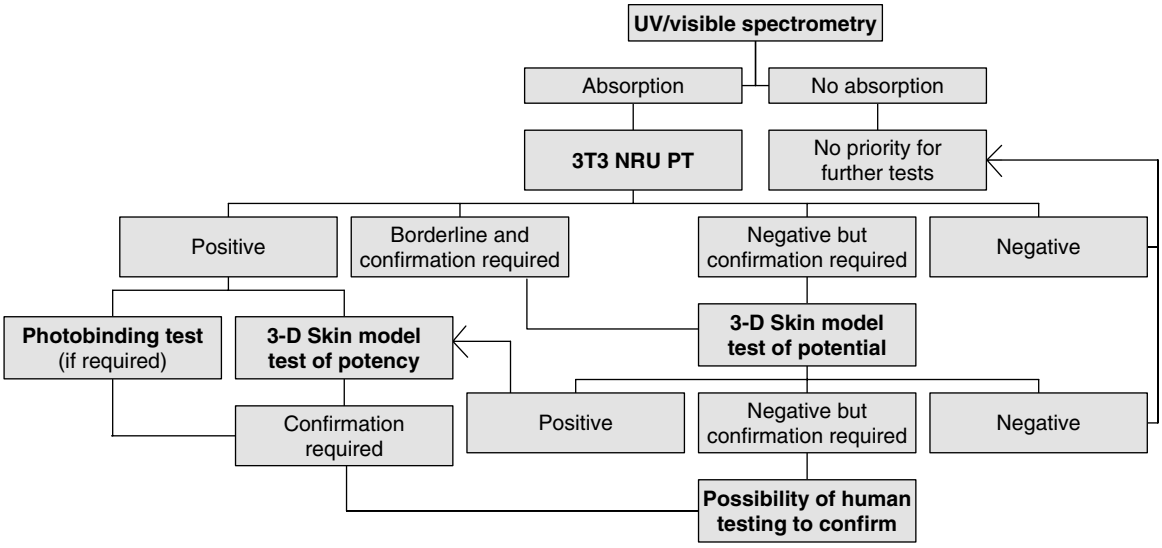


FIGURE 61.2 An *in vitro* testing strategy for the assessment of phototoxic hazard.

testing strategy suitable for this purpose. As an essential requirement for phototoxicity is the absorption of light by a test material, the initial assay is measurement of a UV/visible absorption spectrum to identify absorption at relevant wavelengths (>300 nm). OECD guidelines state that if the molar extinction/absorption coefficient of a chemical is less than 10 L $\times$ mol^{-1} $\times$ cm^{-1}, the chemical is unlikely to be photoreactive and need not be tested in the 3T3 NRU phototoxicity test or any other biological test for adverse photochemical effects (OECD, 1997; OECD, 2004). Another similar practical cut-off for absorption is if the absorption of a 1% solution using a 1 cm path length (A 1% 1 cm) is less than 1.0, then similarly this would not be considered as significant (Lovell, 1993; Lovell and Jones, 2000). This is useful where the molecular weight of a substance is not known or for the consideration of extracts/mixtures. If a substance demonstrates significant UV/visible light absorbance, then it may have potential for phototoxicity and should be tested. The primary test of choice would be the validated NRU PT which has the potential to detect photoirritants and also most photoallergens and photogenotoxins. If a substance is negative in the NRU PT, then this is good evidence that it does not have phototoxic potential and should not require further testing (Spielmann et al., 1998a,b). Examples have been published of the testing of several types of ingredients such as surfactants (Benavides et al., 2004), fragrances (Nam et al., 2004), and essential oils (Dijoux et al., 2006).

In risk assessment practice, a further confirmatory test may be desired to add to the weight of evidence demonstrating absence of hazard prior to marketing the substance. Further testing would also be advisable where a substance gives a borderline result of probable phototoxicity in the NRU PT. In this case, further testing using a 3-D skin model assay would be recommended (Jones et al., 2001). The advantages of 3-D skin models in providing a system more similar to the human skin *in vivo* are given earlier. Toxicity (and phototoxicity) to human skin is affected by penetration rates through the stratum corneum. In general, penetration rates of substances through *in vitro* skin models when measured are greater than that of human skin (Ponec et al., 1990; Regnier et al., 1992; Michel et al., 1995; Doucet et al., 1998, 1999). Therefore, 3-D skin models would be considered as more sensitive to insult than human skin *per se* and the lack of phototoxicity of a substance in such a model is good evidence that it would not present a phototoxic hazard to human skin *in vivo*. An example of the use of a 3-D skin model assay to further investigate borderline results in the NRU PT for a personal product ingredient has been published by Jones and co workers; in this study, the material was found to be nonphototoxic to the skin model *in vitro* (Jones et al., 2003). Alternatively, a borderline result may be confirmed as positive by the 3-D skin model.

A positive result for a substance in the NRU PT (or a 3-D skin model) provides evidence of possible phototoxic hazard (which would feed into the overall risk assessment of the substance). If further information on the nature of the hazard is required, then a photobinding test for photoallergy may be carried out. At this stage, further information can also be obtained by carrying out a dose–response for phototoxicity in a 3-D skin model. This could give information on possible potency by comparison with intended use levels and in particular by comparison with a phototoxin of known *in vivo* potency (bearing in mind the probable greater sensitivity of the skin models than human skin *in vivo*). The final step in the testing strategy is the possibility of testing in a human clinical trial provided that sufficient information has been obtained for ethical testing, a situation which seems unlikely except for essential novel pharmaceutical preparations; for consumer personal care and household products, evidence of photopositivity is most likely to lead to cessation of product development rather than clinical testing. However, it should be noted that 3-D skin models also lend themselves to the testing of formulations as they allow application to the

stratum corneum surface (Medina et al., 2001). It has been demonstrated that formulations spiked with a known phototoxin can be identified using a 3-D skin phototoxicity test (Jones et al., 2001). A 3-D skin model phototoxicity assay could, therefore, be useful as an interim step as part of the risk assessment process prior to any human testing.

61.2.3 Future Prospects

The authors are not aware of any significant current progress being made in the deployment of *in vitro* phototoxicity tests, particularly in relation to risk assessment for human health.

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62 Photoirritation (Phototoxicity) Testing in Humans

Francis N. Marzulli and Howard I. Maibach

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62.1 DEFINITION

Photoirritation (phototoxicity) has been defined as a nonimmunologic sunlight-induced skin response (dermatitis) to a photoactive chemical, with the response being likened to an exaggerated sunburn (Marzulli and Maibach, 1970). The photoactive chemical may reach the target tissues either after direct application or indirectly via the blood stream, following ingestion or parenteral administration. The skin response is characterized by erythema and sometimes edema, vesiculation, and pigmentation.

62.2 SCREENING

Chemicals that are phototoxic are usually activated by the ultraviolet (UVR) portion of the sun's radiation, which involves wavelengths in the range 280–400 nm. Screening tests for evaluating phototoxic potential should therefore begin with an examination of the test chemical under UVR. Fluorescence under UVR examination suggests that the chemical may be photoirritating and may require further investigation. Additional screening can be performed with *in vitro* tests (Nilsson et al., 1993). Finally, human tests should not be undertaken prior to familiarization with and performance of animal tests.

62.3 SPECTRAL MEASUREMENTS

The basic unit of radiant work energy emitted by a source is the joule (J). One joule delivered over 1 s is 1 W of radiant power. Radiant power, or irradiance, is reported in watts per square meter (often W/m^2) and radiant exposure is reported in joules per square meter (often J/cm^2) skin. Optical radiation is measured with a radiometer. Other details about spectral measurements are given in the chapter on spectral equipment for photobiology.

62.4 PRECAUTIONS

The American Conference of Government Industrial Hygienists (1988) has established that total irradiance upon unprotected skin or eye should not exceed 1.0 mW/cm^2 for exposure times greater than 16 mm and should not exceed 1.0 J/cm^2 for exposure times less than 16 mm. Recommendations for other exposure periods are also given.

A report by the Commission Internationale de l'Eclairage (CIE) states that a review of a considerable amount of data suggests that the damage risk for UVR at 290 nm appears to be about 100 times higher than that at 320 nm (McKinlay and Diffey, 1987). The damage risk at 320 nm is about 10 times that at 340 nm and about 100 times that at 400 nm. These findings have resulted in a modification of one of the traditional terms (UVA) employed by the photobiologist to describe portions of the solar spectrum that are accorded special biologic attention. Besides UVA (320–400 nm), UVB (280–320 nm), and UVC (<280 nm), we now have UVA1 (340–400 nm) and UVA2 (320–340 nm). Other values for UVA, UVB, and UVC are given by the Commission de l'Eclairage (1970).

The upper atmosphere screens out some UVB radiation; however, in 1982, the National Bureau of Standards reported that radiation down to 286 nm was measured at Gainesville, Florida (National Bureau of Standards, 1982).

Urbach (1989) emphasized the importance of spectral distribution as compared with intensity of the UVR source. He found that a 4% reduction in total UVR produced by doubling the filter thickness (Schott WG 320) from 1 to 2 mm

produced a 50% decrease in erythema effectiveness because of change in spectral distribution of UVB.

62.5 EXPLORATORY STUDIES

Tests that rely on simple equipment can provide a satisfactory starting point. Marzulli and Maibach (1970) used this approach in identifying bergapten (5-methoxypsoralen) as the principal phototoxic component of oil of bergamot. The mouse, rabbit, guinea pig, and other mammalian species can be used for exploratory work, with humans as the ultimate test subjects. The rabbit has a large area of the back, which can be divided into four test sections, enabling a reduction in the number of test animals required.

A source of UVR with over 90% of the UV radiation (300–400 nm) output at 365 nm was provided by a Hanovia Inspectolite (no longer available). When used with no. 16125, type EH-4 bulb, red purple Corning 7-39 (5874) filter, and frosted glass cutoff at 290 nm, total output at 10 cm from the source is about 3000 μW/cm^2 and about 1900 μW/cm^2 at 15 cm.

The test chemical (0.05 mL) is applied to skin, and after 5 mm it is irradiated for 25–40 mm (animals–humans) at a distance of 8–10 cm. The skin is examined for erythema and edema at 24 and 48 h and again at 7 days. Positive controls (using 0.01% 5-methoxypsoralen or 8-methoxypsoralen in 70% ethanol) and negative (vehicle) controls are similarly exposed for comparison.

Appropriate modification of the aforementioned basic scheme is needed for investigating orally administered chemicals, such as nonsteroidal anti-inflammatory drugs.

62.6 ADDITIONAL FUNDAMENTALS

Duplicating the sun's spectrum in the laboratory has been one of the challenges posed to scientists and engineers during the past 20 years.

Experimentalists currently engaged in photobiologic work need to report the source and output of radiation used in their experiments. They should employ sources with output of UVA and UVB and should specify exposure time and distance of source to the skin. The UVA should be about 10 J/cm^2 and the UVB about 0.1 J/cm^2.

Irradiance from the UVR source can be measured with a UV radiometer at an appropriate distance. For correct readings, the radiometer is calibrated by the supplier, with the intended source. Irradiance is measured in mW/cm^2; dose in J/cm^2; and exposure time (t) in minutes.

Urbach (1989) reported that:

> The sun emits a polychromatic continuum of different wavelengths; low pressure fluorescent sun lamps emit a continuum mainly in the UVB, or mainly in the UVA; high-pressure mercury arcs provide discontinuous line spectra; and high intensity solar simulators, based on xenon, xenon-mercury or doped tungsten may mimic solar UVR, but require special filtration to shape the UVB spectrum and remove intense visible and infrared radiation. The age of the lamp, temperature of the bulb or arc, and age of filters will influence both the spectral power distribution and irradiance. (p. 178)

62.7 LAMP SOURCES

In the United States, General Electric Co. (Baltimore), Sylvania Corporation (Danvers, Massachusetts), Solar Light Co., Inc. (Philadelphia), and Elder Pharmaceuticals (Bryan, Ohio) market lamps with UVA and UVB outputs. Xenon Corp. (Wilmington, Massachusetts) manufactures solar simulators. Optronics (Orlando, Florida), Eppley (Newport, Rhode Island) and G. Gamma Scientific (San Diego, California) market radiometers. United Detector Tech. (California) calibrates radiometers. Schoeffel Co. (New Jersey) is a source for detectors and simulators (Anderson, 1986).

62.8 CONCLUSIONS

The literature on phototoxicity contains a large assortment of data involving a wide variety of compounds, test methods, equipment, and results. Maurer (1987) reviewed and studied the published findings on humans and animals and voiced concern about the complexities of phototoxicity testing and the limitations of the predictability of test results. Nilsson et al. (1993) submitted to the Organization for Economic Cooperation and Development (OECD) a proposed standard protocol for topical and systemic phototoxicity testing in guinea pigs, which was evaluated and found satisfactory in a collaborative study involving six different laboratories. The method provides test details such as experimental design, irradiation sources, and scoring. The proposed method is similar to the one employed by Lovell and Sanders (1992). It is suggested that these animal test methods be employed as part of the familiarization experience that is needed prior to exposing humans to UVR in laboratory tests.

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63 Measuring and Quantifying Ultraviolet Radiation Exposures

David H. Sliney

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63.1 INTRODUCTION

Quantitative phototoxicology requires appropriate dosimetry. The important concepts, terminology, and units of optical dosimetry required in photobiology are all too frequently not fully appreciated in experimental studies, leading to needless misinterpretation and error. The objective of this chapter is to familiarize scientists working in photodermatology with the basic concepts related to light measurement and light source characterization that are necessary for reproducible scientific tests. Hopefully this chapter will encourage the reader to look differently at the optical source being used and how correctly to express the exposure dose. Phototoxicological studies require knowledge of the optical and radiometric parameters of ultraviolet optical sources and geometrical exposure factors. This knowledge is required to accurately determine the irradiances (dose rates). In performing any phototoxicity study, it is imperative that the spectral characteristics of the optical source must be known. For a specific photobiological action spectrum, different light sources delivering the same optical power can produce completely different dermatological effects if the sources have differing spectra (Figure 63.1). Indeed, a photodermatologist will choose a specific ultraviolet (UV) source to match best a given biological action spectrum (if known) to achieve the greatest efficiency in delivering a photobiologically significant dose. Different applications require different light sources and a variety of measurement techniques may be in order when attempting to conduct different types of studies. Photochemical interaction mechanisms are normally most pronounced at short wavelengths (UV) where photon energies are greatest, and also will be most readily observed for lengthy exposure durations (Sliney and Wolbarsht 1980; WHO, 1994).

The concepts of a photobiological dose evolved from applying standard physical measures of exposure (radiant exposure) and exposure rate (irradiance) used by physicists (Sliney, 2007). The basic physical measures (termed *radiometric quantities*) were modified by applying the laws of photochemistry to employ action spectra, and by applying rules as the Bunsen–Roscoe law (i.e., the rule of reciprocity). Because of the reciprocal relationship of irradiance (dose–rate in W/cm^2) and exposure duration (in seconds, s) to achieve a threshold photochemical radiant exposure (dose in J/cm^2), the cumulative exposures from either a single lengthy exposure or repeated exposures within a given time (usually a few hours) will be additive. Prior to any discussion of light sources and measurements, it is important to consider those physical quantities and units useful in photobiology. These quantities are termed radiometric (and spectroradiometric), and they should not be confused with the photometric system of measurements (lumens and candelas) used by lighting engineers. Radiometry is the science of measurement of optical radiation. Photometry is the science of measuring visible radiation, that is, *light.* Radiometric quantities such as radiant power—used to describe the output power (not electrical input power) of a source in watts (W)—must be used to describe and quantify ultraviolet radiation (UVR). Photometric quantities such as

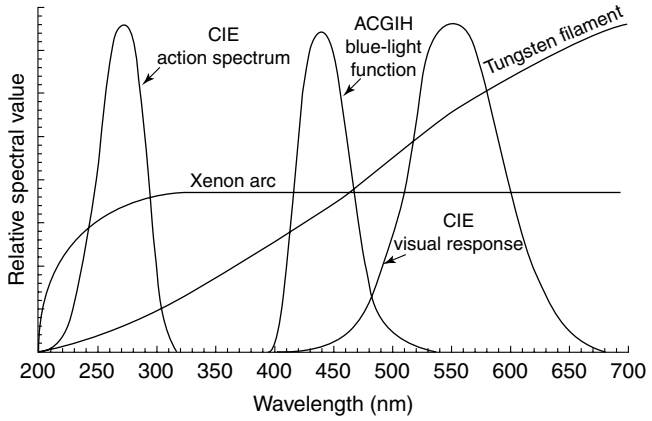


FIGURE 63.1 The effect of different light source spectra for delivering an effective dose relative to the same action spectrum. Three sources with the same total radiant power output are not equally effective in delivering a photobiologically effective dose. Indeed, the narrow emission of a red LED does not even overlap the action spectra shown (ACGIH, 2002).

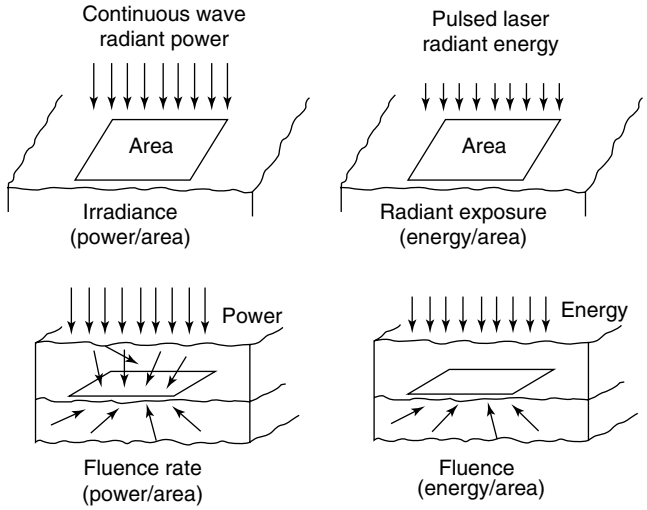


FIGURE 63.2 Radiometric quantities and units used in photodermatology.

luminous power—used to describe the luminous output of a lamp in lumens (lm)—are based upon the relative visibility of different spectral sources as seen by a human "standard observer." In illuminating engineering, light levels are spectrally weighted by the standard photometric visibility curve, which peaks at 550 nm for the human eye.

To quantify a photochemical effect, it is not sufficient to specify the number of photons-per-square-centimeter (photon flux) or the irradiance (W/cm^2) since the efficiency of the effect is highly dependent on wavelength. Generally, shorter-wavelength, higher-energy photons are more efficient. Photometric quantities are hybrid quantities that are defined by the action spectrum for vision—a photochemically initiated process. Photometric quantities have no value in photobiology except in describing visual processes and retinal photochemistry.

Unfortunately, since the spectral distributions of different light sources vary widely, there is no simple conversion factor between photometric (either photopic or scotopic) and radiometric quantities. This conversion may vary from 15 to 50 lm/W for an incandescent source, to about 100 lm/W for a xenon arc, to perhaps 300–400 lm/W for a fluorescent source (Sliney and Wolbarsht, 1980). The fraction of radiant energy in the ultraviolet is much less still. Although the action spectra for erythema in normal human skin has been published by many investigators (Anders, 1995; Berger et al., 1968; Coblentz et al., 1931; Diffey, 1975; Diffey, 1982; Everett, 1965; Freeman, 1966; Hausser, 1928; Lukiesh, 1920; McKinlay et al., 1987; Parrish et al., 1982), the action spectra of photosensitized responses appear to be quite imprecise or even unknown (Diffey, 1982; Fitzpatrick et al., 1974; Parrish et al., 1982; Parrish et al., 1978; Urbach and Gange, 1986; Urbach, 1969). This suggests the need to specify the spectrum of the light source used as well as the irradiance levels if one is to compare experimental results from different studies.

63.1.1 Radiometric Quantities

The following radiometric quantities may be used in photodermatology, and are briefly summarized here (Figure 63.2):

A. *Irradiance* (surface dose rate) and *radiant exposure* (surface dose) are units specifying power or energy incident on a plane. As shown in Figure 63.3, these quantities are the dose rate (irradiance) and exposure dose (radiant exposure) that are the most fundamental dose quantities used in all of photobiology. The units most commonly used are W/cm^2 and J/cm^2, respectively.

$$1 \text{ W} = 1 \text{ J/s}.$$

B. *Fluence rate* and *fluence* are used in some very sophisticated studies, where the internal surface dose with backscatter is included. These quantities are used correctly most often in theoretical studies, but these terms are frequently misused to mean irradiance and radiant exposure because the units of W/cm^2 and J/cm^2 are the same.

C. *Radiance* (irradiance per solid angle) is an important quantity used by physicists in specifying a source. This quantity limits the ability of lenses and reflective optics in concentrating a light source. For example, a xenon-arc lamp has a very high radiance and its energy can be focused to produce a very high irradiance on a target tissue. In contrast, a fluorescent lamp tube has a much lower radiance, and its energy cannot be focused to a high concentration. The units are W/(cm^2·sr).

D. *Radiant intensity* (power per solid angle) is used to indicate how collimated a light source really is. Although useful for specifying searchlights, it

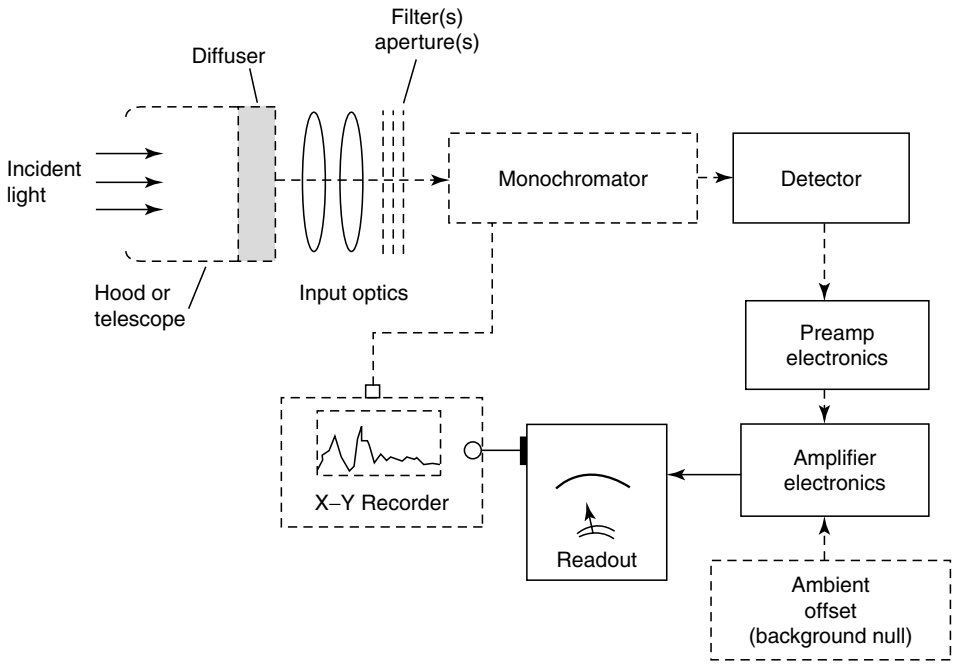


FIGURE 63.3 Short-wavelength spectral cutoff filters are used to check monochromators and the presence of stray light.

normally has very limited use in photobiology. The units are W/sr.

E. *Spectral quantities* (units per wavelength) are used for specifying the energy, power, or irradiance per wavelength interval. When calculating a *photobiologically effective dose*, the spectral quantity must be multiplied by the action spectrum. Examples: spectral radiant power, spectral irradiance, and spectral radiant exposure. The units for each quantity are modified by adding "per nanometer," for example, W/cm^2 becomes W/(cm^2·nm).

F. *Photon (quantum) quantities* (units of photons) are used primarily in theoretical studies, and in photochemistry. In this case, the radiant exposure is specified in photons/cm^2 and irradiance is specified in photon/(cm^2·s).

63.1.2 Spectral Band Notations

When considering UVR bioeffects, it is useful to employ the convention of the International Commission on Illumination (CIE) for spectral bands. The CIE has designated 315–320 to 400 nm as UV-A, 280 to 315–320 nm as UV-B, and 100–280 nm as UV-C (Sliney, 2007). Wavelengths below 180 nm (vacuum UV) are of little practical significance since they are readily absorbed in air. The 308 nm UV wavelength is therefore in the UV-B spectral region. UV-C wavelengths are more photochemically active, because these wavelengths correspond to the most energetic photons, are strongly absorbed in certain amino acids and, therefore, by most proteins (Grossweiner, 1984; Smith, 1988), whereas UV-B wavelengths are less photochemically active, but are more penetrating in most tissues. UV-A wavelengths are far less photobiologically active, but are still more penetrating than UV-B wavelengths. UV-A wavelengths play an interactive (sometimes synergistic) role when exposure occurs following UV-B exposure (Willis et al., 1972). UV-B radiation has been shown to alter enzyme activity in the lens (Tung et al., 1988).

It is very important to keep in mind that these photobiological spectral bands are merely a useful "short-hand" notation, and they can be used to make general (but not absolute) statements about the relative spectral effectiveness of different parts of the UV spectrum in producing effects. The dividing lines, while not arbitrary, are certainly not fine dividing lines between wavelengths that may or may not elicit a given biological effect. One should always provide a wavelength band or spectral emission curve for the UV source being used and not rely totally on these spectral terms. There are also many authors who use 320 nm rather than the CIE-defined dividing line of 315 nm to divide UV-A and UV-B. Some authors also may divide the UV-A band into two regions: UV-A1 and UV-A2, with a division made at about 340 nm.

63.1.3 Radiometric Principles

There are a number of general principles that help to avoid errors in dosimetry. Probably the most important principles are

1. *Bunsen–Roscoe law.* It specifies the reciprocity of photochemical reactions. Within the recovery time of the system (generally a few hours for erythema and photokeratitis), the product of the dose rate (irradiance) and the exposure duration results in a

constant dose (radiant exposure) required to elicit a given effect. Therefore, a very high dose rate from a very intense source can result in the same effect within a very short time as a less intense source can produce in a much longer time.

2. *Critical photon energy.* To produce a photochemical effect in tissue, normally only one photon will alter one molecule when we examine the process at the molecular level. For this reason, the individual photon energy must be sufficient to alter the molecule, or else the interaction results in imparting energy to the molecule that will probably result in vibrational motion (and in a temperature rise at the macroscopic level). For this reason, the long-wavelength cut-off for the action spectrum is normally fairly, sharply pronounced. This also makes the measurement of a rapidly changing action spectrum quite difficult unless very narrow wavelength bands are used.
3. *Inverse-square law and the "rule of ten."* For most sources that are not highly collimated (such as a laser), the well-known "inverse-square law" aids in extrapolating the irradiance to different distances from a small optical source. While the inverse-square law is strictly true only for a geometrical point source, the "rule of ten" allows us to apply it to practical lamp sources. You will have a very small error (less than 10%) if you begin your extrapolation at a distance of at least 10 times the diameter of the source. For example, if one has a small mercury arc lamp with an arc size of 3 mm, then at 10 times the 3 mm, that is, at 30 mm, one would expect the measured irradiance to decrease inversely as the square of the distance beyond that point. For example, if that lamp source was specified to produce an irradiance of 10 mW/cm^2 at 20 cm, then at twice that distance the irradiance would drop to (1/2^2) 1/4th its value, or to 2.5 mW/cm^2. At four times that distance, the irradiance would drop to (1/4^2) 1/16th the value of 10 mW/cm^2, or only 625 Î¼W/cm^2. Thus, without making detailed measurements at each distance, a wide range of dose rates can be established by merely moving to different distances from the source.
4. *Law of conservation of radiance (brightness).* This principle derives from the physical law of conservation of energy. Basically, it states that no matter what optical focusing lenses one employs, the focal irradiance cannot exceed a value limited by the brightness of a source. Arc lamps and the sun are of nearly equivalent radiance, whereas a tungsten source has a much lower radiance. Lasers have by far the greatest radiance of any source, and their rays can be concentrated in a very high focal irradiance. The use of larger collecting optics can increase the size of the irradiation zone, but not the irradiance.

63.2 OPTICAL RADIATION SOURCES

There are many different types of optical sources that can be used in photodermatology. Even the sun has been employed in some studies, but the sun's changing spectrum from hour to hour makes it difficult to use for reproducible exposures. Artificial sources are therefore almost always used in laboratory studies, and these are generally high-intensity lamps.

Broadband, polychromatic sources deliver much more irradiance than narrow band sources. With a monochromator or narrow-band filters, one can achieve nearly monochromatic (one-wavelength) sources of light or UVR by sacrificing the total power available. By the Bunsen–Roscoe law, the same exposure dose would require a much longer time to deliver. Nevertheless, to determine an action spectrum for a given photobiological effect, one is forced to take this approach. If the action spectrum is known, or if one wishes simply to simulate sunlight with a *solar simulator*, one can achieve a threshold exposure dose in a far shorter time with broadband sources. The continuous light sources used most frequently are

1. tungsten lamps and tungsten–halogen lamps;
2. high-pressure gas discharge lamps, for example, deuterium lamps;
3. arc lamps, for example, xenon, or mercury–xenon high pressure lamps;
4. low-pressure discharge lamps, generally used for "line" sources.

Other continuous sources, such as light-emitting diodes (LEDs), lasers, and synchrotrons, have generally not been of much value, although lasers have been used for rapid determination of action spectra. Pulsed sources, such as flashlamps, could be employed to achieve stepped doses, but this is rare.

The greatest pitfalls with regard to the optical exposure in phototoxicity studies relate to poor *source characterization*. Unfortunately, to properly characterize a light source is quite difficult. Some of the factors that should be considered are as follows:

1. *Lamp envelope and filtration.* The spectral filtration of the glass or quartz envelope determines how much short-wavelength UVC and UVB is emitted by the lamp. A quartz envelope transmits down to approximately 180–200 nm and gives the broadest spectrum of UVR. Most glass envelopes filter out wavelengths below 310–320 nm. In addition, through the use of short-wavelength cutoff filters such as those shown in Figure 63.3, the relative contribution of UVB and UVA can be controlled. Since the terrestrial solar spectrum has a cutoff between 295 and 305 nm (depending upon time of day), filters with cutoffs in this region are used in solar simulators.
2. *Source size and distance.* Through the use of the inverse-square law, it is possible to produce a range

of irradiances for a given experiment. However, if the source size is rather large (e.g., a UVA "blacklight" reflectorized spotlight), the irradiance will not decrease rapidly with increasing distance from the lamp. If a high-pressure arc lamp is used, the arc size may by only a couple of millimeters in diameter, and the inverse-square law would apply within a few centimeters from the source.

3. *Source uniformity.* The uniformity of the source can affect the uniformity of the irradiance patter, particularly if the source is focused. If the UV source is not readily visible, a fluorescent card may be used to visually examine the irradiance pattern at the point of the subject's exposure. An aperture can be positioned over the site of greatest uniformity to achieve reproducible exposure doses.
4. *Source stability.* Tungsten–halogen (and tungsten) lamps generally provide the most stable optical sources with little fluctuation with time. However, because relatively little UVR is emitted from these types of lamps (tungsten–halogen lamps emit more UVR because of higher operating temperature), a slight change in supply voltage can result in much greater changes in UV output. A stabilized power supply may therefore be necessary. Arc lamps typically produce some degree of flicker, which can be averaged over most practical exposure durations. The temporal stability of lasers varies greatly with the type used.
5. *Aging characteristics.* The aging characteristics of different sources vary considerably, but all sources will change with extensive use. It is therefore best to have at least an inexpensive monitor to measure the relative output of the source from day to day.
6. *Temperature and environmental sensitivities.* The sensitivities of different light sources to environmental changes, for example, with temperature, are generally not great, but if the laboratory environment does change, the UVR monitor should be used to check for possible changes. The greatest change of output with temperature occurs during lamp warm-up. Mercury discharge lamps are particularly noted for this, and a 5-min warm-up period is generally advisable; one can check for this with the UVR monitor.
7. *Radiant efficiency.* The radiant efficiency of different light sources varies greatly with regard to the UV radiant power emitted for a given electrical input in watts. Arc lamps are probably the most UV-efficient sources, but require special power supplies. Radiofrequency power supplies for arc lamps can also create substantial electromagnetic interference and disable electronic instruments and nearby computers. Gas discharge lamps such as sun-lamps require less expensive power supplies, or they can be operated at main voltages. Lasers are notoriously inefficient, for example, an argon laser emits less than 0.1% of the electrical input power; however, the monochromatic purity of the laser cannot be equaled.

63.3 UV MEASUREMENT

63.3.1 Types of UVR Measurement Instruments

There are a variety of different instruments that can be used to measure UV radiant power or irradiance. They vary greatly in cost and complexity (and accuracy). Depending upon the application, a relatively inexpensive UV monitor may suffice, but the user must be aware of the limitations of the instrument.

Instruments are often grouped by the detector type used (e.g., a photomultiplier tube, a photodiode, a photofluorescence detector, or a thermal detector). The photo-multiplier tube (PMT) is by far the most sensitive; hence, it is used in instruments where very low irradiances must be measured, as with monochromators to measure the spectral distribution of a lamp.

Most simple instruments have a "broadband" spectral response, which may cover just a band in the UV or may include the visible. These meters use a photodiode. A special type of UV-B detector employs a fluorescent screen that is sensitive only to the short-wavelength UVR, and a photodiode measures the visible fluorescence, which is proportional to the actinic UVR. This seemingly complicated method is used to greatly reduce out-of-band response—a severe problem in UVR radiometry. Thermal detectors are seldom used, because these respond to all wavelengths from UV to infrared, although for certain calibrations they may be useful for an absolute measurement. Apart from arc lamps, most optical radiation sources such as the sun or general service fluorescent and incandescent lamps used for illumination emit only trace quantities of UVR. Indeed, the UV-B or UV-C emission is normally less than 0.1–1% of the total radiant power output. For this reason, attempting to measure UV-B in the presence of so much longer-wavelength radiant energy presents an incredible challenge. If a special UV lamp is in use, this may not be a problem, and a simple, less expensive instrument may suffice.

In addition to the broadband instruments just described, it will be necessary to use a *spectroradiometer* to measure the spectrum of the incident UVR. These are rarely inexpensive or simple instruments, but are essential to obtain the spectrum of the lamp source being used, unless the spectrum can be obtained from the manufacturer. All spectroradiometers have three fundamental elements: input optics, a monochromator (prism or diffraction grating to disperse the spectrum), and a detector. Spectroradiometers are too complex to describe in any detail here, and the reader is referred to any number of references (Kostkowski, 1997; Sliney and Wolbarsht, 1980). It is important, nevertheless, to be familiar with the importance of using appropriate input optics for an experiment. The input optics are the lenses, optical

fibers, diffusers, and apertures used between the point of measurement and the entrance slit of the monochromator. Frequently, substantial errors are introduced by a failure to understand the limits of the input optics in achieving a good "cosine response," and uniform spatial response. Some of the response factors that should be taken into account are the linearity of response with increasing light input, the nature of the entrance aperture and whether it is smaller than the irradiation area being measured, the cosine response, the temperature response, and the wavelength range. The most challenging problem of monochromators is the rejection of out-of-band radiation, or "stray light." A double-grating or double-prism monochromator is far better in stray light rejection than a single element monochromator. Again, this problem may not be significant if one is using a UVR source with little or no visible or infrared output.

63.3.2 Performing the Measurement

Prior to using any UV meter or spectroradiometer, one should attempt to perform a general characterization of the instrument. It is most helpful to measure several types of sources where there exist published output and spectral data with which to compare. Since many radiometric errors originate from a failure to adequately appreciate the geometry of exposure, be sure that the input optics are appropriate for the measurement.

Calibration of the instrument should be traceable to a national laboratory and the type of secondary standard should be noted to see if it is at all similar to the light source you are using. If not, you may wish to inquire of the instrument manufacturer as to any special limitations of your instrument and whether it is appropriate for the task in mind. Some laboratories maintain some limited calibration capability, such as a reference lamp or a reference detector or standard meter. In general, calibration may be "reference source based" or "reference detector based." Examples of reference optical sources are the free-electron laser (FEL) tungsten–halogen lamp, a standard deuterium lamp (for UV-C), or even a laser. Reference detectors generally have a flat response, for example, a pyroelectric detector or a disc calorimeter, although some reference silicon detectors are used in the ultraviolet spectral region. In any calibration procedure, the source specifications must be understood, and the uncertainty in the final calibration should be determined. The determination of the contribution of all errors in the process frequently is termed the "error budget."

Before purchasing an instrument, one should hopefully know what level of measurement accuracy the project requires. As noted earlier, a simple UVR monitor may suffice if one is using a well-characterized lamp and there is little visible light to produce a stray-light problem. It is also important to estimate the level of measurement uncertainty that you can live with. For example, in a pass–fail situation, where one desires to determine if the measured level exceeds a limit of *X,* and the measurements are typically at 50% of *X*, one can use an instrument with a 20–30% uncertainty, without being too concerned of passing a source that emits too much UVR.

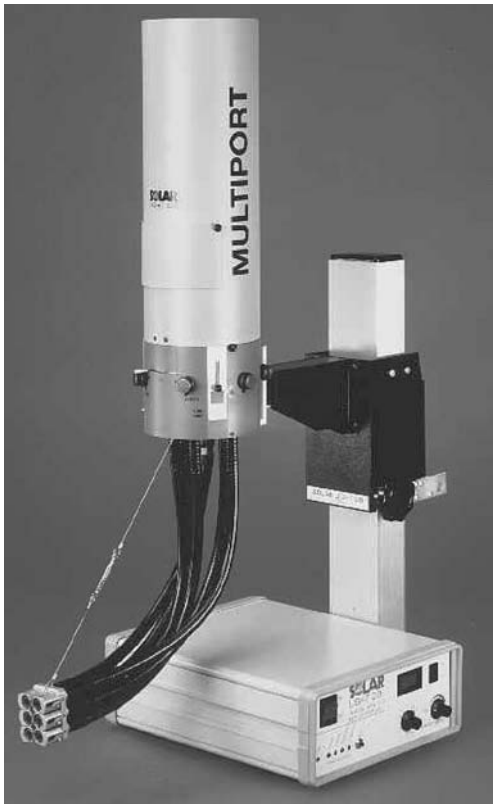


FIGURE 63.4 Multiple-port UV irradiator. In this unit, an arc lamp source is filtered to simulate sunlight and multiple liquid light guides are used to provide more than one irradiation site with stepped irradiance (dose rate) values. (Photo, courtesy of Solar Light Company, Philadelphia, PA.)

However, if the level is typically 95% of the limit, a very accurate measurement with an uncertainty less than 5% is required. For phototoxicity studies, one should be pleased if one can achieve a 20% accuracy in irradiance measurements.

For the instrument manufacturer to adequately assess the accuracy and stability of the instrument, a thorough study of the system and the sources and components of the system is required—a challenging task. In the end, the individual investigator must rely on the reputation of the manufacturer and the experience of colleagues with different instruments. Do not expect a simple instrument to perform the task required of a much more sophisticated instrument.

Special exposure equipment exists for phototoxicity testing, and the manufacturer provides the radiometric output characteristics and spectrum. Figure 63.4 shows one such instrument that features a multiple-port fiber delivery system to allow multiple simultaneous exposures of the skin at different irradiance levels.

63.4 GENERAL BIOPHYSICAL AND PHOTOBIOLOGICAL FACTORS

The purpose of this section is not to add another review of photobiology, but to consider the dosimetry required for adequate comparison of UVR effects being determined in

various studies. While the great majority of investigators in phototoxicology have made UVR measurements designed to characterize the exposure conditions, the calculated levels of exposure that have been published in different studies often vary by more than a factor of 10 among studies employing the same light sources. This chapter will hopefully reduce that problem.

For photobiologically induced injury of tissue occurring after prolonged exposure (i.e., greater than 10 s in the visible and UV-A; greater than at most 1 ms in the UV-B and C), it is generally agreed that the mechanism of injury is initiated by a photochemical event, rather than a thermal event. Two key factors distinguish a photochemical process from a thermal process. Thermal injury is a *rate process* and is dependent upon the volumic absorption of energy across the spectrum. By contrast, any photochemical process will have a long-wavelength cutoff where photon energies are insufficient to cause the molecular change of interest. A photochemical reaction will also exhibit reciprocity between irradiance (exposure dose rate) and exposure duration. Repair mechanisms, recombination over long periods, and photon saturation for extremely short periods will lead to reciprocity failure. For the lengthy exposures characteristic of phototoxicity studies, it is difficult to know what effective exposure time to use for an exposure calculation. Irradiance E in W/cm^2 times exposure duration t is equal to the radiant exposure H in J/cm^2, that is, the exposure dose.

$$H = E \cdot t \tag{63.1}$$

Since most photobiological effects are photochemically initiated, it is necessary for the UV or visible photons to penetrate to the target molecules *the chromophores*, to trigger the photochemical event. Therefore, the action spectrum for a given effect in the skin is actually not only the *in vitro* action spectrum for the target molecules, but as altered by the spectral transmission of the overlying tissue, such as the stratum corneum. UV-C wavelengths are strongly absorbed in proteins, are very photochemically interactive, and have the least penetration into biological tissue. In this regard, the ArF excimer laser wavelength of 193 nm provides the extreme case of a wavelength shown very clearly to produce damage to the cell wall without effectively penetrating to the nuclei of many cell types.

The *biologically effective irradiance* E_{eff} from an optical source is obtained by a mathematical weighting of the spectral irradiance $E_{\hat{\imath}»}$ and the normalized action spectrum $S(\hat{\imath}»)$, which is unitless:

$$E_{\text{eff}} = \sum E_{\lambda} \cdot S(\lambda)\Delta\lambda \tag{63.2}$$

where $\Delta\lambda$ is the spectral interval. The spectral region of summation would be from approximately 200 nm (or where the lamp envelope permitted emission) to at least 400 nm over the full range of the action spectrum. This effective irradiance is very useful, and some instruments have a spectral response function designed to mimic a common action spectrum such as erythema.

With regard to photocarcinogenesis, it is generally agreed that only a very narrow UV-B wavelength band is generally considered very effective in producing skin carcinogenesis (Cole et al., 1986; Cunningham-Dunlop et al., 1977; Sterenborg and van der Leun, 1987)—and for that matter, severe sunburn (Hausser, 1928) and cataractogenesis in humans (Sliney and Wolbarsht, 1980). Even though we are concerned with immediate effects in the skin, a study of the delayed effects upon both dermal and ocular tissues from UVR can provide a deeper insight into the potential for biological effects upon the skin, as well as the eye in this wavelength region (Cullen et al., 1984; Kopecky, 1979; Sliney, 1972; Sliney, 1986; Sliney, 1987a; Sliney, 1987b; Sliney et al., 1991; Stenson 1982; Takise, 1988; Taylor et al., 1988; Kurtin and Zuclich, 1978; Lavin et al., 1982; Luckie et al., 1930; Marshall et al., 1986; Olsen and Ringvold, 1982; Pitts, 1973; Pitts, 1974; Pitts et al., 1977a; Pitts et al., 1977b; Pitts and Tredici, 1971; Puliafito et al., 1987; Riley et al., 1987; Ringvold, 1980a; Ringvold, 1980b; Ring, 1983; Ringvold and Davanger, 1985; Ringvold et al., 1982; Zuclich and Connolly (1976), Zuclich (1989).

In *phototoxicity studies,* the exposure site can be limited for topical photosensitivity tests. Relative thresholds and altered action spectra can be tested on the same subject at different skin sites for comparison. In this case, the multiple-port exposure system (shown in Figure 63.4) can be useful for more rapid testing. In such tests, it is presumed that the spectrum eliciting the phototoxity is sunlight, and this is one reasonable approach where an in-depth study of action spectra is not possible. However, it should be remembered that although the action spectrum of a photosensitizer might be the same as its absorption spectrum *in vitro*, as with some drugs, the photosensitizer my interact with proteins or DNA and produce an altered erythema spectrum. Obviously, the nature of the interaction in tissue and the resultant action spectrum can influence the results of a photosensitivity test depending upon the light source used.

63.5 DETERMINING ACTION SPECTRA

In phototoxicology, it is frequently necessary to determine an action spectrum for the photosensitization. Initially, a very crude action spectrum may suffice. This can be accomplished by the use of several band-pass filters or short-wavelength filters to determine the band that appears to be most photosensitizing. However, for the purposes of basic photobiology or if the action spectrum is later to be used by engineers to determine the efficacy of different lamps in eliciting a response, a much more refined action spectrum will generally be necessary. In comparing action spectra obtained in different laboratories, scientists are frequently puzzled at apparent differences. The sources of these differences generally arise from the use of different means to obtain the monochromatic light. A tunable continuous-wave laser will provide the most precise and accurate result (Anders, 1995), but this is generally

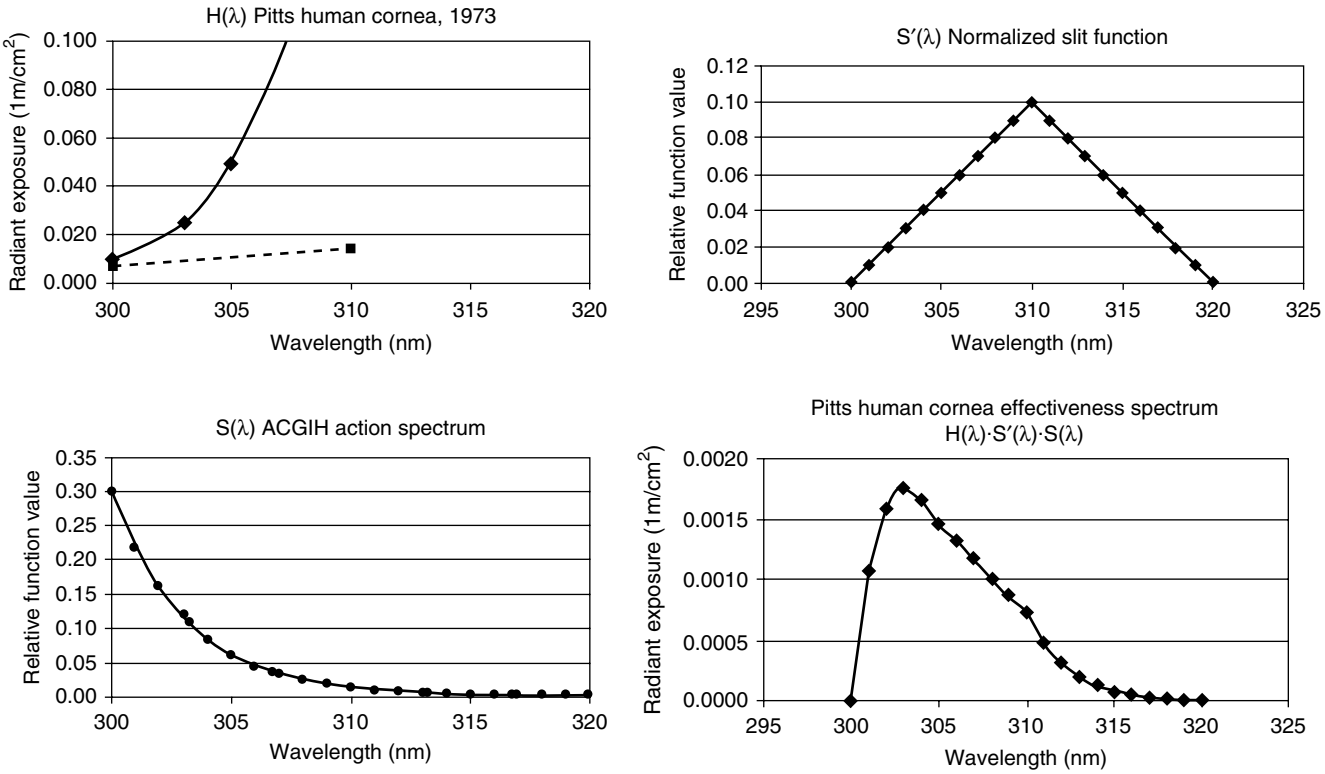


FIGURE 63.5 The expected slit function for a monochromator set to provide a bandwidth of 10 nm at a wavelength of 310 nm. The actual resultant beam of UVR has some energy from 300 to 320 nm. If a true action spectrum varies greatly within the region, the effective wavelength equivalent to a truly monochromatic source (e.g., a laser) would be shifted from 310 nm. For example, a DNA-related action spectrum effective wavelength could be as low as 303 nm.

unavailable, and one must employ a number of narrow-band spectral filters to sample the spectrum or a tunable diffraction-grating monochromator to provide the selected narrow-bands to sample the region of interest (Diffey, 1975; Sliney and Wolbarsht, 1980; Young and Diffey, 1985). The choice of monochromator and selection of a sampling spectral bandwidth can greatly impact the resulting action spectrum (Sliney, 1998), and photobiologists frequently overlook this effect.

63.5.1 Choice of Monochromator Bandwidth

When determining action spectra using monochromators and a broadband UV source, a very narrow bandwidth is essential to obtain an accurate spectrum for further applications. This, however, is a very difficult challenge to the experimentalist. Typical UV sources that have been used in photobiological threshold studies are low-pressure mercury lamps with filters or xenon-arc (or mercury-xenon-arc) high-pressure lamps with a grating monochromator. The spectral filters or the monochromator isolates the desired wavelengths. The disadvantage of the low-pressure lamp is that only a limited number of wavelengths are available. While the xenon arc monochromator is continuously tunable, it suffers from poor resolution, as the slit widths must be great enough to pass enough power. When *analyzing any published action spectrum*, it is necessary to consider the spectral bandwidth for all data and to consider also "stray radiation" and the sources of stray radiation in the instrument; for example, stray light scattered from the gratings in monochromators, leaks in radiometric housing.

The slit function is the spectral power distribution of radiant energy emitted at the given wavelength set on the monochromator (Kostkowski, 1997; Sliney and Wolbarsht, 1980). For a perfect grating monochromator, the shape of the slit function is triangular, as shown in Figure 63.5. Although it would always be desirable to employ a high-resolution monochromator slit-width, such as 0.5 or 1.0 nm, the throughput is so low that a threshold exposure could require hours. In practice, much larger band-pass values of 5–10 nm are therefore used. Threshold data are frequently plotted against the center-wavelength of each spectral band pass; however, significant plotting errors can be introduced into the true action spectrum when the slit width of the monochromator is not accounted for (Anders, 1995; Chaney and Sliney, 2005; Diffey, 1975; Kostkowski, 1997; Sliney and Wolbarsht, 1980). To derive a true action spectrum from the low-resolution threshold data obtained from monochromator studies, a mathematical convolution is required.

If the target molecule, or chromophore, is a protein or DNA, the most effective dose will normally come from the shorter wavelengths (i.e., those before the center wavelength of the slit function), since these will contribute much more to the effective dose than the longer wavelengths in the rapidly changing 300–320 nm region.

Published photobiological threshold data are frequently plotted as an action spectrum with relative response versus wavelength. The accuracy and resolution of the action spectrum are significantly influenced by the choice of the monochromatic source and the number of data points obtained. Measurements of UV action spectra for erythema or photokeratitis can be especially challenging in the 300–320 nm spectral region, as the UV hazard action spectrum, $S(\lambda)$, changes quite rapidly in this region, that is, the limits increase by an order of magnitude between 303 and 310 nm, and another order of magnitude between 310 and 320 nm.

When the slit function covers a region where the true action spectrum varies greatly with wavelength, it is difficult to arrive at the "true" effective wavelength for proper plotting of the data points of the action spectrum. No monochromator really achieves a perfect, triangular slit function, but instead there is a "skirt" at the base of the triangle. For example, if the slit function in Figure 63.5 applies to taking a data point for photokeratitis (corneal UV damage) at a monochromator setting of 310 nm, the 310 nm threshold would have an enormous error. At 300 nm, the slit function had at least a 1% value, the effective wavelength shifts noticeably to the shorter wavelengths, because the true action spectrum, simulated by $S(\lambda)$ at 300 nm, has a value 20 times more effective than at 310 nm.

In an effort to show the impact of monochromator bandwidth on threshold data, Chaney and Sliney studied the impact of bandwidth on effectiveness spectra of photokeratitis studies. They recalculated, assuming the bandwidths specified in the literature and the revised action spectrum was remarkably steeper than that published by the authors of the original study (Chaney and Sliney, 2005).

63.6 CONCLUSION

While all reports of photosensitization contain some quantitative exposure information, important spectral information is often lacking. The careful development of an action spectrum for each type of interaction is an important research goal when dealing with a new target molecule. Radiometric and spectroradiometric measurements of lamps permit one to calculate the effective exposures.

When one considers the very small, but biologically significant, fraction of UVR in natural sunlight, one is struck by the fact that even fluorescent lamps and other lamps used in general indoor illumination also have a fraction of UVR that may be biologically significant. The enormous importance of considering both spectral and geometrical factors in phototoxicity experiments cannot be overstated.

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64 Determination of Subclinical Changes of Barrier Function

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64.1 INTRODUCTION

The epidermis serves as a barrier to the outside world. Through its external layer, the stratum corneum (SC), the epidermis plays a mechanical protective role and minimizes the exchange of materials between our body and the environment. One of the major functions of the SC layer is to prevent excessive evaporation of water from the viable cell layers. Removal or alteration of the SC results in a significant increase in the rate of water loss from the skin [1]. The SC mainly consists of protein-enriched corneocytes embedded in a highly organized lamellar bilayer of lipids [2]. It is generally assumed that this cutaneous barrier is disrupted through intercellular lipid disorganizations, lipid removal, or protein alterations [3]. The "barrier" maintains SC functionality by preventing fluid loss and by minimizing the penetration of exogenous substances.

However, the epidermis is not an inert membrane since it undergoes a continuous proliferation–desquamation process. Basal cells at the junction with the dermis continuously divide and move toward the surface. During this migration, the cell morphology changes and lipids and proteins are both synthesized and modified by enzymatic reactions. During the final stages of the proliferation process, lipids are excreted into the intercellular space to provide a unique structure that defines the skin's permeability properties [4]. These lipids as well as proteinic bridges called desmosomes play a role in ensuring intercellular cohesion. Desmosomes are found between the keratinocytes, and corneosomes or corneo-desmosomes are found between corneocytes [5]. In the nonpalmo-plantar SC, the density of these "bridges" significantly decreases in the upper layers of the SC and they only remain at the periphery of the corneocytes to weaken the cohesion between cells [5]. In the most external layers of the SC, sometimes defined as the stratum disjunctum, proteolytic enzymes degrade the corneosomes, and isolated corneocytes detach and are released from the surface of the skin [6]. Cell turnover, from division to desquamation, lasts for about 4 weeks in normal conditions. If any of these maturation or renewal steps is impaired, for instance by contact with skin irritants, the desquamation rate and pattern and the water impermeability barrier properties are affected [7]. Corneocyte release does not occur anymore in the form of isolated cells, but rather as clusters of corneocytes forming scales or flakes. Moreover, water is not retained by the SC anymore, but evaporates at a much increased rate.

Human skin irritation is classically evaluated by clinical (visual or tactile) scoring. Bioengineering methods measuring skin surface capacitance, color, transepidermal water loss (TEWL), and blood flow have been introduced over the last 25 years to get more objective measurements and have been widely used [8–14]. But the identification of the effect of substances of low or subclinical irritation potential still remains problematic in some instances. Also, these bioengineering techniques have been of great help to detect and quantify the "invisible" irritation effects [15]. Changes in the electrical properties of the SC are very early predictors of skin surface alterations [15–17], and evaporimetry measurements

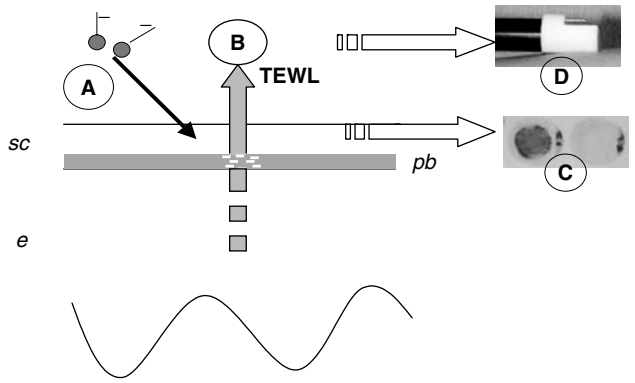


FIGURE 64.1 Skin barrier subclinical alterations. Anionic surfactants interact with the stratum corneum (A), which can be assessed by squamometry (C). This interaction causes an alteration of the permeability barrier (*pb*) of the stratum corneum (*sc*) and as a result, transepidermal water loss (TEWL) increases (B), as measured by evaporimetry (D), and *e* is the epidermis.

allow early discrimination between surfactant-based products in irritation tests. These measurements detect differences earlier than those observed visually by trained evaluators [18].

An increasing interest has been observed for techniques focusing on the SC. Cellophane and related tape forms used to remove and analyze the SC have been developed with numerous applications: quantifying SC desquamation [19,20], barrier function perturbation [21,22] or percutaneous penetration [23], observing histological changes [24,25], and testing cosmetics and drugs effects [26,27]. To evaluate nonerythematous irritant dermatitis, squamometry (SQM) appears to be a sensitive complementary method to conventional skin color, TEWL, and hydration measurements [28–33].

This chapter mainly focuses on two types of methods to evaluate subclinical barrier changes: TEWL, which is the most conventional technique, and SQM, which has found broad application in testing surfactant–skin interactions. TEWL mainly measures the effect of the alteration of the barrier function, while SQM investigates one of the origins and prior interactions with the SC that have caused the barrier to be altered (Figure 64.1).

Finally, combining tape stripping and TEWL measurement has given rise to a new concept, the measurement of the barrier strength [34], which will also be discussed.

Another usual cause for skin barrier disturbance is an alteration of the intercellular lipids' high organization. The effects of surfactants on the SC lipids and their consequence are covered in chapter 46 of this book.

64.2 MEASUREMENT OF SUBCLINICAL BARRIER CHANGES BY EVAPORIMETRY (TEWL)

The barrier's integrity seems to be a key factor in determining the responsivity of the skin to external aggressions. Patients with atopic dermatitis have been reported to have elevated TEWL values [35], and are more readily irritated by detergents than nonatopic subjects [36]. Similarly, in a normal population, subjects with the highest TEWL values were reported to be the most susceptible to surfactant irritation [37,38]. In experimental test models, skin is sometimes compromised by subirritant treatment with sodium lauryl sulfate (SLS) to predamage the cutaneous barrier [39]. In such cases, TEWL is enhanced and skin responsiveness to mild irritants increased (Paye, personal data). In all these situations, evaporimetry measurements proved to be very sensitive in detecting damages to the skin barrier associated with reduced skin protection caused by external irritants.

Measurement of water loss from the skin has also been widely used to investigate and compare the irritation potential of products (mostly surfactant-containing) entering into contact with the surface of the skin [18,36,40]. This is the case for hygiene products such as soaps, shower gels, and foam baths as well as for a multitude of detergent products such as hand dishwashing liquids, household cleaners, or even fabric cleaners. In occlusive patch tests with surfactant-based products, the SC barrier function is impaired before any erythema is induced [18]. This is expected as occlusion is known to affect the barrier properties of the skin by itself [41]. Furthermore, the test product first encounters the SC and alters the barrier function before reaching deeper skin layers to induce erythema. When very mild products are tested under such conditions, it is typical to observe impairment of the barrier function in the absence of an erythematous reaction. In contrast, for very irritating products or substances, the skin barrier can be very quickly damaged. The resulting inflammation occurs immediately and predominates over the TEWL readings. Evaporimetry measurements of SC alterations are thus mostly appropriate for comparing very mild products to each other when no or minimal erythema is induced. Clinical evaluation of the erythematous reaction is often preferable when one wants to compare harsh products. TEWL can be used to monitor barrier damage as well as to follow the recovery of the barrier integrity during the repair phase following a skin challenge [42]. There is thus a great deal of evidence supporting the use of evaporimetry measurements to study cutaneous alterations at a subclinical level. However, owing to the high sensitivity of the measurements and the numerous factors that can affect the water evaporation rate of subjects, a lot of caution must be considered to obtain accurate measurements.

64.2.1 Principle of TEWL Measurements

Water diffusion from the skin may involve several components: the sweat gland activity, the evaporation of water from the surface of the skin, and the evaporation of body water through the SC. This last component is usually referred to as the TEWL and, provided that the other two components are controlled and minimized, it can be used to monitor changes in the water permeability barrier function of the SC. Several instrumental methods have been investigated to measure TEWL from local skin sites.

64.2.1.1 Open-Chamber Evaporimetry Measurements

Historically, water evaporation from the skin was measured using closed chambers, in which water evaporating from the skin was collected and quantified. However, those initial systems had many drawbacks and major progresses were made since the description by Nilsson [43] of a method based on the estimation of the vapor pressure gradient immediately adjacent to the surface of the skin in an open, ventilated, chamber. Using this kind of chamber, the skin is exposed to normal ambient air, and the development of a warm and humid microclimate at the measurement site is avoided.

In the probe of the measuring device, two vapor pressure–sensitive sensors are placed at a specific distance from each other. When the probe is placed horizontally to the surface of the skin, these sensors are able to determine the vapor pressure gradient above the skin surface. This gradient is then translated into the amount of water crossing the SC per hour and square meter of skin surface (g of water/h m^2). This value, measured below the sweating temperature and in nonstressful conditions, is relatively low (usually between 4 and 10 g/h m^2 depending on the body site) when the skin barrier is intact, while it can grow up by 10–30 times when the barrier is severely damaged.

Well-known, but not exclusive, instruments for measuring TEWL are the ServoMed Evaporimeter® and the Courage & Khazaka Tewameter®. Both are based on the same principle and have been described and compared [44]. More recently, a third instrument has been described, the DermaLab® system (Cortex Technology, Hadsund, Denmark), which has a TEWL probe. The DermaLab system has been compared to the Evaporimeter, and very good agreement has been observed [45]. It is an open-chamber system and measures a water vapor gradient. Temperature and humidity at the level of each of the two sensors are constantly displayed during measurements, and the system can be linked to a computerized program [46].

The ServoMed Evaporimeter is probably the one that has been most widely used to measure TEWL, as it is the first one in the market place. It is easy to handle and to carry, and provides instant measurements of the TEWL from the skin. Its main advantage, other than the quality of the instrument, is its large use around the world rendering comparison of data between laboratories quite easy. One of the main disadvantages is the time required before the displayed value stabilizes (usually between 30 s and 1 min depending on the extent of barrier alteration); an internal integration system of the values for 10, 20, or 30 s exists to minimize those fluctuations and it is also possible to link the Evaporimeter to a computerized program (cyberDERM®, Media, Pennsylvania, or ServoMed, Kinna, Sweden) to register the evolution of the measured values and integrate them on a certain period of time after TEWL has reached stability. This makes the use of this instrument much easier and the precision of the measurements much greater. More details can be found in Ref. 47.

The Tewameter, which is most popular nowadays, has also been designed such that it is easy to use and carry, and directly provides integration values and graphic representations of the evolution of the measurements on a screen. Information about temperature and humidity values of the probe is also recorded during the measurement and could be of some importance for the accuracy of TEWL measurement [48]. The sensitivities of Tewameter and Evaporimeter have been compared, both of them having some advantages depending on the extent of the barrier alteration.

64.2.1.2 Closed-Chamber Measurements

The major drawback expressed for the open-chamber evaporimeters is the great sensitivity to the ambient air flux coming from door opening, ventilation from the air conditioning in the measurement room, panelist breathing and talking, etc. To get rid of such constraints, closed-chamber systems have been reinvestigated and adapted to the requirements of modern bioengineering instruments [49,50]. The VapoMeter® (Delfin Technologies Ltd, Kuopio, Finland) [50,51] has been developed and commercialized as a portable and pocket-size closed-chamber system for TEWL measurements. The instrument, which is based on the use of a humidity sensor placed in the closed chamber, claims to provide quick (not more than 10 s measurement) and reliable measurements without interfering with the normal evaporation and not be affected by ambient or body-induced airflows [51]. The chamber is passively ventilated between measurements for a period that is automatically controlled based on the residual relative humidity in the chamber. As the VapoMeter measures a level of humidity in the chamber and not a water flux, the measurement is said to be independent on the position and angle of the probe, so that the instrument may be used on various body sites [52]. This is, however, not agreed by everybody [53]. A comparative study has been conducted by De Paepe et al. [54] to validate the model and get a comparison with the Tewameter. A good correlation was obtained between both instruments, with a constant feature of measuring higher TEWL values with the Tewameter than the VapoMeter. This may be due to the fact that the VapoMeter displays a mean value based on the initial and progressively increasing rate of humidity within the chamber that does not represent a steady-state measurement [49]. Owing to these lower recorded values, the VapoMeter also looks less sensitive than the Tewameter to discriminate between low differences in water evaporation from the skin.

Another popular closed-chamber system is the Biox Aquaflux® [55]. Like the open-chamber systems, it is based on a diffusion gradient measurement inside the probe. This probe is, however, closed on the top by a condenser that continuously removes water vapor from air in its vicinity by freezing. One sensor is located at mid-height of the chamber and the second one in the condenser. Different caps may be adapted on the probe for different purposes, but each requires new calibration. There are caps of 3, 7 (standard one), or 15 mm of diameters; slotted caps for scalp measurement between the hairs; tube caps for difficult-to-reach sites; or soft contact caps for measurement on "nonflat" hard surface

like fingernails. The advantages are the same as those of the Delfin Vapometer with the possibility to take measurement under various environmental conditions and angles. Good correlation with data generated with the DermaLab and the Tewameter have been obtained [56,57].

64.2.2 Cautions for TEWL Measurements

Owing to the sensitivity of the TEWL measurements and the numerous factors that can affect water evaporation, a lot of parameters must be carefully controlled for evaporimetry measurements. Those parameters reviewed in previous publications [58–61] may be related to individuals (e.g., extreme age, anatomical site, menstrual cycle periods, circadian rhythm, and sweating characteristics); environmental factors (e.g., ambient temperature or humidity, and air convection); measurement or instrument variables (e.g., calibration, temperature of the probe, and positioning of the probe); or test variables (e.g., occlusion, moisture in the vehicle for leave-on products, and delay between product application and measurement). For the closed-chamber systems, great care must also be taken to avoid humidity saturation in the closed chambers by using a short measurement time or sufficient rest time between successive measurements, low relative humidity conditions in the measurement room, and correct zeroing of the instrument between repetitive measurements. Depending on the instrument, these controls may be autoregulated by the device itself.

For all these reasons, several guidelines and recommendations for a proper assessment of TEWL and a proper interpretation of results have been published [54,59,60].

When those guidelines are strictly followed, TEWL measurement is unequivocally an excellent method to measure the effect of subclinical changes in the integrity of the skin barrier.

64.3 ASSESSMENT OF SUBCLINICAL BARRIER ALTERATIONS BY SQUAMOMETRY

Few human *in vivo* studies have been described relating to assessing subclinical surfactant-induced irritation [15,28,30,31]. In those studies that have, open models were used to better approximate consumer surfactant use. Our goal was to determine whether we could differentiate between various surfactant solutions in terms of their skin surface effect at a subclinical level. SQM provided some insight into changes of irritation (suberythematous irritation) not readily discerned with clinical readings and bioengineering instruments.

64.3.1 Squamometry Methodology

The use of the adhesive D-SQUAME® disc as a harvesting method for the superficial desquamating layer of the SC has been discussed in detail [61], and guidelines for their analysis have been published by the EEMCO (European group for the efficacy measurement of cosmetics and other topical products) [62]. SC tape strippings may be rated by visual examination after placing the sample on a black bottom, by weighting, by optical measurement with or without specific staining, by image analysis, by protein assay, and by morphometry [62–64].

In this chapter, we focus our attention on an optical measurement method and on morphometric changes of stained SC tape strippings. SQM is a noninvasive, protein-dependent, colorimetric evaluation of the level of alteration in the corneocyte layer collected by clear adhesive-coated discs. Xerotic and irritant changes in the SC can also be quantified [63,65]. The discs are applied onto the skin under controlled pressure. A short application time (15 s) enables the harvesting of the superficial corneocytes (superficial SQM) and a long application time (1 h) enables the collection of a thicker layer of corneocytes (deep SQM) [62,63]. The discs are stained for 30 s with a solution of toluidine blue and basic fuchsin in 30% alcohol (polychrome multiple stain, PMS; Delasco, Iowa), applied to the surface, and gently rinsed in water. Tape strippings are placed on a transparent microscope slide, which is itself placed on the calibration white plate of the Chromameter® (Minolta, Japan). As described by to Pierard et al. [65], measurements of the color of the samples in the L*a*b* mode are made using a reflectance colorimeter. Calculation of the Chroma C* values is done after $(a^{*2}+b^{*2})^{1/2}$. This parameter combines the values of the red and blue chromacities, predominant colors of the PMS. The Chroma C* value has been shown to be related to the amount of SC harvested in xerotic situations [65]. The calculation of the colorimetric index of mildness (CIM), with CIM=L*–C*, was performed [63], where L* is a measure of the luminance. A trained person scores the discs with a microscope at ×20 magnification according to the following scoring scales [15,66]:

Intercorneocyte cohesion:

0 = Large sheet
1 = Large clusters + few isolated cells
2 = Small clusters + many isolated cells
3 = Clusters in disruption, most cells isolated
4 = All cells isolated, many cases of lysis

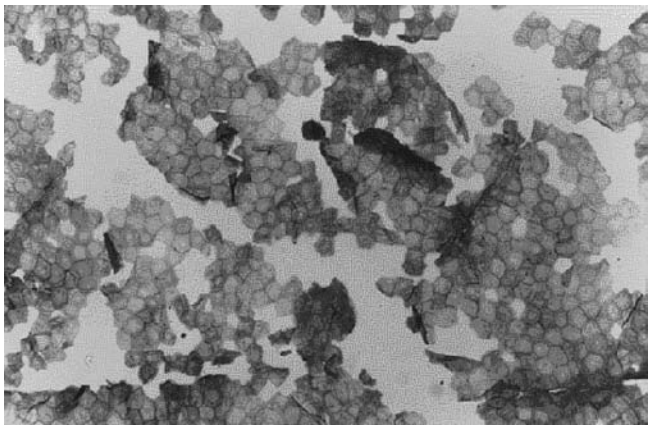


FIGURE 64.2 Illustration of a stained disc with PMS.

Amount and distribution of dye found in cells:

0 = No staining
1 = Staining between cells or slight staining in cells
2 = Moderate staining in cells
3 = Large amount of dye in cells, but uniform
4 = Important staining in all cells, often with grains

An illustration of a stained disc is presented in Figure 64.2. This methodology has been used in the studies mentioned Section No. 64.3.2.

64.3.2 Subclinical Alterations of the Stratum Corneum with Surfactant

After a single occlusive application (24-h patch test), low concentrations of SLS (0.5% in water) can cause irritation, dryness, tightness, and barrier alterations [42,67,68]. These occlusive tests are, however, too severe to observe subclinical damage, since erythema and skin barrier alterations predominate. Furthermore, occlusion plays a significant role in the barrier alterations that can hide the effect of the test product at that level. In typical use, the consumer's contact with the surfactant is brief and via hand washing or personal cleansing and repetitive. The open application model becomes relevant when phenomena, such as dryness and subclinical (i.e., nonvisible) irritation, especially cumulative faint alterations of the skin surface barrier, are induced. SLS can induce subclinical skin damage in a repetitive open-application test method (exaggerated model hand wash) as well as in short-exposure patch tests. Analysis of the skin surface via SQM offers a unique way of measuring skin changes when traditional methods do not. It also permits exploration of subclinical surfactant irritation and SC alterations [28,69].

In two of the studies [28], we performed four successive SC tape disc strippings on the dorsal hand to estimate the penetration pattern of the surfactant solution in terms of the skin barrier damage effect measured via SQM. Data on dye fixation per cell revealed differences between SLS solutions at 0.75 and 2.25% w/w in water as early as the first day (out of four treatment days) on the upper two tape strips. Thereafter, differences appeared on all days for each of the four tape strippings successively collected on the same site. In such an application mode, dryness manifested itself first in the uppermost SC (two most superficial tape strippings) before appearing in the lower layers (third and fourth tape strippings) after more washings. Overall, there was an increased staining of the tape strips from the first up to the fourth day. Thus, the 24-h patch test and the open test are disparate: skin response to an SLS challenge indicates inflammation and dryness in the occlusive patch, while only faint, nonvisible alteration due to surface interactions occurs in the open assay.

A single skin challenge with an irritant is a momentary reflection of skin susceptibility with little bearing on the cumulative effect of irritation or the associated repair mechanisms. An occlusive application of surfactant enhances the penetration of the irritant into or through the SC and permits to investigate the interaction of the surfactant with deeper skin layers than in an open test. More importantly, the occlusive application test is not appropriate to study and conclude on the effect of an irritant on the skin barrier function. In real life, consumers are exposed to surfactants mainly through skin washing. The contact time of the surfactant with the skin is thus short, nonocclusive, and followed by a rinsing phase. The surfactant can thus in most cases only interact with the SC without reaching deeper layers.

In test models, SQM allows one to assess irritation effects under more realistic test conditions without causing any overt irritation [70]. To refine the exaggerated hand-washing model [28], we tried to move from exaggerated hand washing to more consumer-realistic conditions. The exaggerated hand-washing procedure was replaced by three daily controlled washes at the laboratory for 5 days, one wash on day 7 after the weekend, and volunteer use at home for 1 week (instructions and gloves were provided for other use of products at home over the test period) [30]. The two compared surfactants were SLS and Sodium Lauryl Ether Sulfate (SLES). Based on the results of this study, SQM was able to document subclinical nonerythematous effects. The data also suggested that differences existed between SLS and SLES at 5% w/w both by Chroma C*, CIM, and microscopic examination of cell cohesion and dye fixation per cell. Even if most differences were observed 1 h after the day 7 wash in the laboratory, the CIM (the higher, the milder) and the Chroma C* (the lower, the milder) statistical analysis also revealed a significant difference between SLS and SLES before the controlled laboratory wash on day 7, which was after volunteers were self-dosed over the weekend. Encouraged by these results, an open assay, using only volunteer washing (whole body wash) at home as usual, was tested. Bioengineering measurements, SQM, and clinical assessments were performed after three washes and after a week's use at home [31]. The conventional techniques of erythema, dryness, capacitance and evaporimetry were not capable of distinguishing between the effects of the two surfactants on skin after the first three washings. SQN clearly and significantly showed that SLS was more damaging to the SC than SLES by Chroma C* measurements and microscope assessment of corneocytes cohesion loss and dye fixation per cell. Even if it was expected that protected sites (i.e., forearm) could be more discriminating in a home-use assay, SQM proved also to be sensitive enough to differentiate between the effect of surfactant solution on any skin site after as few as the three first home washes. Details have been reported in Ref. 71.

64.3.3 Conclusion

An open application method seems essential, particularly in the detection of subclinical skin surface alterations occurring during test methodologies relevant with the normal consumer practice. Within this respect, it is crucial for the clinician to have, at his disposal, very sensitive techniques used to evaluate the SC. SQM appears to be a robust and facile complementary method to conventional skin color, TEWL, and

hydration measurements, particularly in the detection of subclinical alterations. Section 64.3 only briefly described a few applications of SQM in open test models; however, several other uses have so far been published where SQM has been used to investigate xerotic conditions [62,65]; the interaction of body cleansing products [63,72], shampoos [73], or dishwashing liquids [74] with the skin; differences of corneocytes cohesion between sensitive and nonsensitive hands of subjects [75]; and the effect of skin barrier protectants [76] or topical drugs [77]. With so many potential applications and its extension of use in many different groups, SQM seems to represent a chosen method for investigating the effect of irritants on SC integrity.

64.4 MEASUREMENT OF THE SKIN BARRIER STRENGTH

More recently, a combined use of repetitive controlled tape stripping and TEWL measurement has allowed to define differently the skin barrier properties and to introduce the concept of "skin barrier strength" or "skin barrier integrity" [34]. Controlled tape stripping are collected on a same site and the amount of stripping required to reach a given TEWL value (usually 20 g/h m²) is determined. The higher the number of needed strippings, the better the integrity of the barrier function and strength of the SC. However, a decrease in the number of required strippings suggests an impairment in SC cohesion causing an alteration of the barrier function. Two factors mainly affect the skin barrier strength, the cohesion between corneocytes layers and the thickness of the SC. Regarding SC thickness, a new technique has recently appeared that allows a direct and accurate measurement of that parameter; that is, *in vivo* confocal reflectance microscopy that permits to see inside the skin at the cellular level and, as such, to precisely determine the limits of the SC [78,79].

64.5 CONCLUSIONS

This chapter briefly reviews two different methods used to study the alteration of the skin protective barrier at a subclinical level. With the current trend to develop test models on volunteers that are more respectful of volunteers' skin condition and closer to consumer use habits of the products, it has been mandatory to design assessment methods able to detect "invisible irritation." Evaporimetry measurements, even if not a new bioengineering method, obviously fulfill those requirements to quantify the consequences of the barrier disruption in terms of TEWL. Although easy to use, the method, however, requires a lot of precautions to provide meaningful information. Besides the conventional ventilated open-chamber instruments to measure TEWL, closed-chamber systems have been recently reintroduced with some advantages and drawbacks versus the open chambers. Unlike evaporimetry, SQM does not measure modifications of the barrier functionality. Instead, it documents the alterations caused to the SC, and hence to the cutaneous barrier, by surfactants or other irritants. Since its development [65], SQM has shown numerous applications through several testing procedures, for several types of products, and is now being used in many laboratories.

Targeting two different phases of the skin barrier alteration process, evaporimetry and SQM may be regarded as two complementary tools to follow and understand subclinical skin changes. The proof of such a complementarity has been reinforced by the recently introduced concept of "skin barrier strength" or "integrity." This concept relates to the amount of successive tape strippings necessary to reach a given threshold of TEWL, amount that is dependent on the intercorneocytes cohesiveness within the SC and the thickness of the SC.

This chapter shows that, by using three simple and very sensitive methods, it is possible to follow subclinical changes of the skin barrier function while addressing the three major potential causative mechanisms: a disorganization of the lipidic barrier (evaporimetry), an alteration of the proteins of the SC (SQM), and a reduction of intercellular cohesion within the SC (barrier strength concept, SQM with microscope assessment).

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65 Assessing Validity of Alternative Methods for Toxicity Testing: Role and Activities of ECVAM

Thomas Hartung and Valérie Zuang

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65.1 ORIGIN OF ECVAM, LEGAL BASIS, SHORT HISTORY

European Centre for the Validation of Alternative Methods (ECVAM) is an international reference centre for the development and validation of alternative testing methods aiming at the replacement, reduction, or refinement of the use of laboratory animals in the biomedical sciences, with emphasis on toxicological assessments. ECVAM was established by a communication of the European Commission (SEC 91/1794) to the European Parliament and Council referring to Articles 7.2 and 23 of Directive 86/609/EC on the protection of animals used for experimental and other scientific purposes (EC, 1986). The directive requires that the commission and member states should encourage research into the development and validation of alternative methods, which could provide the same level of information as that obtained in experiments using animals, but which involve fewer animals or which entail less painful procedures. ECVAM became operational as a unit within the EU Joint Research Center (JRC) in 1992. ECVAM's work is focused on the development and validation of *in vitro* methods (e.g., cell and tissue cultures) and of computer modeling based on structure–activity relationships; and on physiological and biokinetic modeling. Actual political needs for ECVAM's core activities are created by the Registration, Evaluation and Authorisation of Chemicals (REACH) and the 7th amendment to the Cosmetics Directive (Hartung et al., 2003).

65.2 ECVAM OBJECTIVES AND STRATEGY

ECVAM pursues its objective to pioneer the process of quality assurance in the life sciences and regulatory testing by:

- Communicating the regulatory needs to test developers
- Guiding the development and optimisation of methods
- Tailoring the validation process and the good practices
- Participating actively in R&D and validation projects
- Developing test strategies and
- Promoting successfully validated tests.

From a strategical point of view, ECVAM has worked out a business plan for the next 10 years. Overall costs of about €150 million over these 10 years were estimated. These costs will have to be shared with industry that now urgently needs validated alternative methods. Structurally, a dense network with stakeholders is in progress of being established under the umbrella of the European partnership for alternative approaches to animal testing, for the development of strategies, the provision of unpublished *in vivo* data, and reference chemicals as well as in-house test methods from industry. Candidate tests for validation are selected either during

taskforces/workshops or submitted by test developers, which is usually done via the ECVAM website, and then prioritized by taskforces.

65.3 PROCEDURE FOR VALIDATION AND REGULATORY ACCEPTANCE

ECVAM operates as a coordinator of international validation studies, a focal point for the exchange of information, the provider of a central database on alternative methods, a centre of public dialogue, and as a prenormative research facility of the JRC. Participation in validation studies requires a running infrastructure and active research maintains a practical and realistic view on science and technology. Furthermore, high-quality research ensures credibility in the scientific community.

Due to the political sensitivity of its duties, ECVAM has its own Scientific Advisory Committee (ESAC) composed of members from all 25 European member states, relevant industrial associations, academic toxicology, animal welfare movement, as well as from other commission services with an interest in the area of alternative methods.

ECVAM has established a wide international network with OECD, with its American counterpart ICCVAM (Interagency Coordinating Committee for the Validation of Alternative Methods) and closely works with other commission services, such as Environment Directorate General (DG), Enterprise and Industry DG, Research DG, and Health and Consumer Protection DG. This network is used for reaching international expert consent, test implementation, and emission of opinions.

A typical validation study lasts 3 years (costs per test are about €300,000) and the subsequent regulatory implementation lasts from 2 to 7 years. Main constraints in the process are the availability of reference substances and animal test data, the need for further optimization of test methods, the duration of financial/administrative procedures, and the long-lasting consensus process of regulatory implementation.

65.4 CHANGES IN THE POLITICAL ENVIRONMENT

65.4.1 The New European Legislation on Chemicals

The European Commission has proposed to harmonize the testing requirements for existing chemicals, for which there is a lack of toxicological data (i.e., chemicals marketed before 1981) and new chemicals, by developing a new system for REACH. The new system will have less stringent testing requirements compared to those imposed by the current legislation on new chemicals, but these will also apply to approximately 30,000 substances, which are currently marketed in volumes higher than 1 tonne per year. The extent of data requirements will depend on the tonnage of chemical produced or imported in the EU. Consequently, this will result in a substantial increase of animal use for the safety assessment of chemicals. Several estimates of the number of laboratory animals required for these assessments, and the costs for performing the tests, have been made. They indicate that several millions of animals will be required, that testing costs will range in billions of euros, and that simply the availability of animal testing facilities will be a limiting factor. Beside the ethical aspects and the public concern, also economical considerations call for the timely development and validation of *in vitro* alternatives.

65.4.2 The Seventh Amendment to the Cosmetics Directive

Much of the scientific work on alternatives, which has been conducted, coordinated, and sponsored in the EU, was strongly pushed by the animal protection community and by the public opinion, who broadly do not support animal testing for cosmetic products. In the EU, the safety of cosmetic products is regulated by Council Directive 76/768/EEC (EC, 1976). Its 7th amendment was approved by the European Parliament and the Council in March 2003 (EU, 2003). It foresees the immediate ban on animal testing for finished products, and a complete ban on animal testing for cosmetic ingredients no later than 6 years from implementation of the directive (i.e., in 2009). Moreover, it requires the immediate marketing ban for new cosmetics (finished products and ingredients) tested on animals where alternative methods validated by ECVAM and accepted by the community exist. It also foresees a complete marketing ban on cosmetics tested on animals within 6 years for some targeted human health effects and 10 years for the repeated-dose toxicities, reproductive toxicity, and toxicokinetics (i.e., in 2013). This latter date can be postponed if by that date no validated alternative methods will be available.

65.5 ECVAM STRATEGIC VISION

From its beginning, ECVAM has been more than the administrator of alternative methods and their formal validation: the field of alternatives requires a proactive role (Figure 65.1), where ECVAM

a. Communicates the needs of the regulatory area to putative developers of new tests
b. Surveys opportunities for new technologies
c. Steers a strategic debate between stakeholders
d. Guides the development and optimization of methods
e. Develops and tailors the validation process
f. Participates in R&D as well as the validation process with its laboratories
g. Collects, compiles, and provides the information on relevant methods
h. Integrates tests into test strategies
i. Promotes tests after validation
j. Pioneers the process of quality assurance in the life sciences

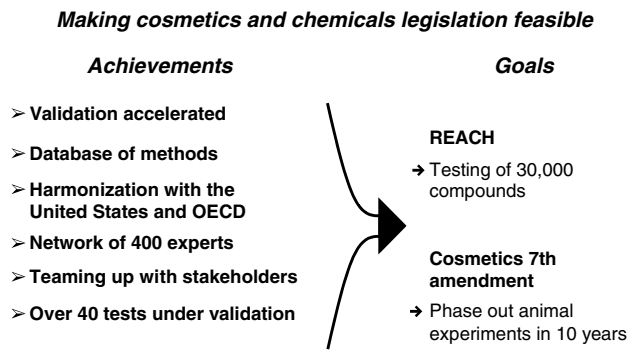


FIGURE 65.1 ECVAM validated alternatives.

Total number	10,700,000	100%
Safety evaluations	1,060,000	10%
Agricultural chemicals	123, 000	1%
Industrial chemicals	136,000	1%
Cosmetics	2,700	0.025%

FIGURE 65.2 Purposes of animal experiments in Europe, in 2002.

These roles have to be regularly revisited in light of the political needs. At this moment, two very obvious areas of concern are REACH and the 7th amendment to the Cosmetics Directive. Beside these, even more impressive with regard to animal consumption (Figure 65.2) are biologicals (16%) if compared to chemicals (1%) and cosmetics (0.025%). Areas like pharmaceuticals (Hartung, 2002) and basic research (Gruber and Hartung, 2004) also deserve further attention.

Highlights of some recent activities related to the ECVAM strategies include:

a. *Communication of regulatory needs to putative developers of new tests.* ECVAM is regularly organizing workshops where several experts are invited to review the current state of the art on various types of alternative methods and to identify the best ways forward. ECVAM task forces are focusing on more tightly defined targets. Task forces have been established in almost all areas of toxicology. The reports and recommendations of ECVAM workshops and the ECVAM task force reports are published in the scientific journal *ATLA* (*Alternatives to Laboratory Animals*). The ECVAM workshop series has celebrated the 50th workshop report (Gennari et al., 2004) in 2004. To date, 56 ECVAM workshop reports have been published.
b. *Surveying opportunities for new technologies.* Following a joint workshop with ICCVAM on validation of toxicogenomics in 2003, a taskforce was established in 2004 and a pilot study was carried out with Affymetrix and Bayer. At this moment a workshop on metabolomics in toxicology is in preparation. Very extensive efforts are spent on (Q)SAR (Worth et al., 2004a,b). By purpose, this activity, a close collaboration between the European Chemical Bureau (ECB) and ECVAM, was put under the umbrella of OECD, where agreement about the principles of (Q)SAR validation and regulatory use is seeked.
c. *Strategic debate between stakeholders.* ECVAM has established a network of about 400 experts regularly working in task forces and workshops. Furthermore, a broad variety of collaborations with all relevant institutions in the field, which aim to bundle stakeholder activities, also exist. For the first time, a European opinion leader meeting was organized in 2004, convening the organizations acting on an European scale to discuss the perception of alternative methods in the (scientific) public and how to improve their image. In close partnership, all ECVAM activities have been opened to the American counterpart ICCVAM. The creation of JaCVAM in Japan, in 2005, has led to first collaborations. With a view to extend this partnership, the establishment of an international council of validation bodies is under discussion.
d. *Guiding the development and optimization of methods.* The EU has already invested more than €170 million into the development of alternative methods by funding respective research. Recently, by funding three large integrated projects, a new dimension of structured development of alternatives was reached (Figure 65.3). These projects, which involve more than 90 institutions and a funding of about €30 million, aim to make available batteries of test, together with the respective test strategies, within 5 years each. ReProTect (Hareng et al., 2005), started in July 2004, deals with the field of reproductive toxicology including endocrine disruption. Noteworthy is that the different tests included into the project for optimization and integration into a test strategy originate not only from the field of alternatives, but also from areas such as mechanistic biomedicine, especially reproductive medicine, pharmaceutical agent discovery, clinical diagnostic, breeding of farm animals etc. These models have never been suggested as alternative methods, since they have different purposes and reflect only partial aspects of the reproductive cycle, but, put together in a conceptual framework, they might allow to build a predictive test strategy. A-Cute-Tox was based on the recommendations of an ECVAM workshop in 2003 (Gennari et al., 2004). The project started in January 2005 and aims to establish an animal-free classification of acute toxicity, substituting for the classical LD_{50} test. Several studies have shown a good correlation of *in vitro* cytoxicity studies and the animal experiment. The project wants to improve this correlation to an acceptable level by outlier reduction. Sens-it-iv started at the end of 2005 with a view to complete the development of animal-free test strategies for skin and respiratory

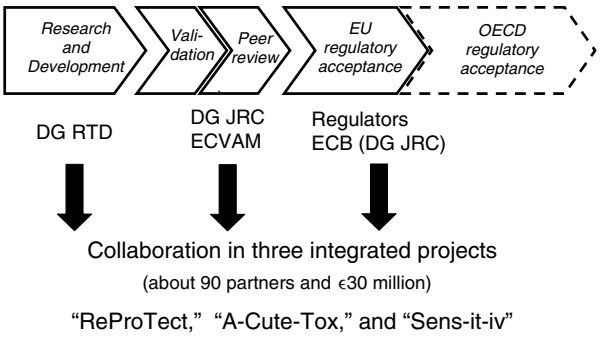


FIGURE 65.3 The new dimension of development of alternative methods.

sensitization. It was based on the recommendations of an ECVAM workshop (Casati et al., 2005).

e. *Developing and tailoring the validation process.* On the one hand, only recently for the first time, international consensus on the role and procedure of the validation process has been reached by the publication of OECD guidance document 34 on the validation and international acceptance of new or updated test methods for hazard assessment. The guidance document mainly reflects the ECVAM principles on validation. On the other hand, several challenges to the validation process as such exist. For example, the enormous efforts involved in the validation of a single test with regard to laboratory work and costs have often been questioned. In light of the need to validate a very high number of tests for the purposes of the chemicals and cosmetics legislations, this aspect had to be reviewed. So far, validation studies did not make use of existing data. However, in some instances if enough data of sufficient quality are already available, such retrospective validation might be an appropriate shortcut in the assessment of validity. Further challenges to the current validation scheme originate from new technologies (pattern-based or "omics" approaches, transgenic animals, *in silico* methods such as (Q)SAR or computer modeling). In response, ECVAM has proposed a modular approach (Hartung et al., 2004), which opens up ways to comply with these new needs. The discussion related to the optimization of the validation procedure, however, continues.

f. *Participation of ECVAM in R&D as well as in the validation process with its laboratories.* As part of the EU Joint Research Centre, ECVAM represents also a place of research and education. Very often, ECVAM has made contributions to the development and optimization of alternative methods. ECVAM's laboratories also allow to participate actively in validation studies any time. Details are given later in this chapter.

g. *Collection, compilation, and provision of information on relevant methods.* ECVAM hosts a database on alternative methods (dbAlm), which provides high-quality information related to alternative methods, including protocols for relevant *in vitro* tests. The major part of this scientific information service will be available online at the end of the year. A valuable resource of documents such as the ECVAM workshop reports is provided free of costs via the ECVAM website (http://ecvam.jrc.it). Another major contribution to the field has been the compilation of inventories of alternative methods, which are currently available. An inventory of 330 pages involving 75 experts in the context of the cosmetics legislation was published as an *ATLA* supplement (Eskes and Zuang, 2005).

h. *Integration of tests into test strategies.* Many of the more complex toxicological endpoints will not be replaced by single alternative methods, but it will be necessary to develop testing strategies based on batteries of tests and their intelligent combination in test strategies. An important element of this is the concept of prevalence of health effects of chemicals (Hoffmann and Hartung, 2005), that is, the actual proportion of chemicals showing a certain toxic property. Furthermore, it is necessary to develop such strategies to analyze the performance of the animal experiment, an effort that has just started, for example, in the field of skin irritation (Hoffmann et al., 2005). Methods of decision theory and evidence-based medicine will be employed to compose and validate finally testing strategies (Hoffmann and Hartung, 2006). In this context, ECVAM is closely involved in the development of test strategies for REACH.

i. *Promotion of tests after validation.* Today, the regulatory implementation of validated tests often lasts longer than the validation process itself. This obvious bottleneck can only be overcome by collaboration with regulators such as the national coordinators of the chemical test guideline program in Europe or at the level of the OECD. However, for this purpose validation has to take the needs of regulators into account, that is, a validity statement is a less scientific judgement but a proof that the method is fit for (regulatory) purpose. Ongoing efforts to create an ECVAM regulatory advisory panel shall further optimize collaboration with regulators and the postvalidation process.

j. *Pioneering the process of quality assurance in the life sciences.* Further in an ECVAM workshop in 1999, an OECD guidance document on good laboratory practice (GLP) for *in vitro* toxicological studies was accepted in 2004 (Figure 65.4). Similarly, a good cell culture practice (GCCP) guidance document (Hartung et al., 2002) was completed recently (Coecke et al., 2005); it sets the minimal standards for quality control of *in vitro* work and will enable an international discussion over the next year.

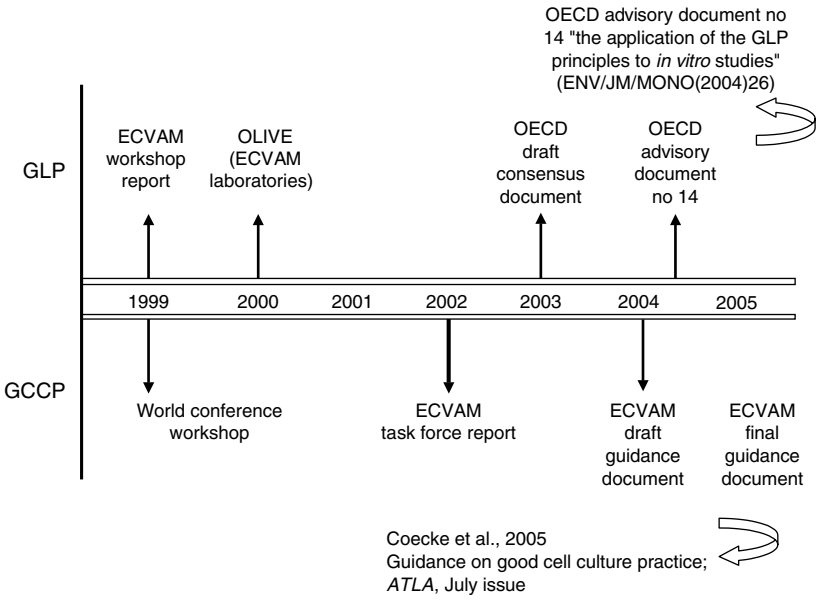


FIGURE 65.4 GLP and GCCP.

In the context of quality control, ECVAM is aiming for further adoption of principles on evidence-based medicine to the field of toxicology (Hoffmann and Hartung, 2006). The establishment of a taskforce on evidence-based toxicology took place in 2004, and currently a conference is prepared for autumn 2007.

65.6 VALIDATED AND ACCEPTED ALTERNATIVE METHODS

Eight alternative methods for chemicals/cosmetics have been endorsed by ECVAM including skin corrosivity, skin sensitization, phototoxicity, as well as embryotoxicty testing of chemicals. Fourteen alternative methods reached scientific acceptance, for example, the safety evaluation of biologicals such as vaccines; and for haematotoxicity, pyrogen testing, and acute fish toxicity. In addition, alternative methods for mutagenicity, acute skin irritation, and chronic toxicity in dogs are currently under peer review by ESAC.

Relevant methods were accepted by both the European Commission (Annex V to Council Directive 67/548/EEC on the classification, labeling, and packaging of dangerous substances), as well as by the test guideline program of the OECD. Three potency tests of biologicals have been accepted by the European directorate for the quality of medicines (*European Pharmacopoeia*).

65.7 ECVAM'S OWN RESEARCH ACTIVITIES

Participation in validation studies requires a running infrastructure. The active participation in validation studies, which ranges from check of Standard Operating Procedures (SOPs) and transferability, to full participation in blinded ring trials under GLP, helps to identify problems of methods and allows to flexibly fill gaps in studies. It also ensures neutrality of the study. Since GLP increasingly becomes a standard for validation studies, ECVAM currently establishes this quality assurance regimen.

Active research maintains a practical and realistic view on science and technology. ECVAM must not become an administrative body, because the validation process is interlinked with developmental aspects. The pipeline of methods to be validated has to be filled actively in interaction with the basic and applied research community. The own research safeguards that this dialogue is realistic and effective.

High-quality research also ensures credibility in the scientific community. This requires visibility and most importantly publications, favorably in higher impact factor journals. Currently, ECVAM researchers publish about 40 scientific papers per year, including prestigious journals such as *Nature* and *Proceedings of the National Academy of Science*.

ECVAM's laboratories have high standards with regard to infrastructure, space, and resources. A very unusual combination of technologies, and expertises (e.g., various *in vitro* technologies, metal toxicology, stem cells) is further amplified by the links to neighboring units and the enormous network of external collaborators. This gives an excellent frame for about 20 PhD students, postdocs, and visiting scientists. All together, a unique integrated approach spanning from basic to applied research and regulatory view is possible. ECVAM's research is uniquely positioned at the gap between basic research and validation. Given the difficult access to animal primary cells at ECVAM and the limitations of cell lines, clear priority is given to human (primary) cells. The emerging stem cell technologies (embryonic and adult) (Bremer and Hartung, 2004; Pellizzer et al., 2005) offer new opportunities, as do the classical accessible human cell sources blood and bone marrow. Cryo-preserved human blood (Schindler et al., 2004) represents a very promising source of standardized cell material without the problem of blood donations. Among the animal cell lines, Balb 3T3 deserve special attention since they are used both for the cell transformation assay

and for the currently validated basic cytotoxicity test. Several projects have led to the identification of prototypic toxins, for example, metal compounds or test substances from validation studies. In some instances, ECVAM holds high-quality *in vivo* and human data. Many of these substances have already been characterized in several standardized *in vitro* systems. This allows synergy linking between projects by testing the same set of reference substances. This will be further increased by the planned establishment of repositories of substances. Currently, a high-throughput testing facility is established jointly as a JRC exploratory research project, which offers opportunities for several projects such as those on acute toxicity (A-Cute-Tox), acute fish toxicity, neurotoxicity, and immunotoxicity. Work on human (stem) cells will always be limited by the number of cells available. Furthermore, organotypic (co)cultures require analyzing individual cells in mixtures, which is also the case for most stem cell–derived differentiated cells. This calls for the establishment of technologies allowing single cell analysis, such as confocal microscopes, Fluorescence Activated Cell Sorting (FACS), cell sorting (all existing at ECVAM), laser scanning microscopy, *in situ* Polymerase Chain Reaction (PCR), laser microdissection, and cell chips. Setting up an array of cutting-edge methods should keep ECVAM also attractive for visiting scientists. As another aspect, the fate of test substances *in vitro*, for example, its solubility, binding to plastic or serum albumin, is routinely not considered. Addressing this might improve the predictive capacity by reducing an uncertainty factor. Similarly, the effect of exposure patterns to test substances *in vitro* has been hardly studied. Several current projects aim to understand the effective exposure of cells *in vitro* and the consequences for toxic responses. Signature-based ("omics") and computational models promise new approaches in several fields. The full integration in all key areas will be important to leverage these technologies. Current projects make use of toxicogenomics and metabolomics (nuclear magnetic resonance [NMR] and mass spectroscopy [MS]).

65.7.1 Good Laboratory Practice—Good Cell Culture Practice

The requirement for carrying out validation studies under standardized conditions, that is, apply GLP and GCCP rules, has been recognized by national and international validation bodies. ECVAM plays a leading role in this process and actively contributes to the drafting of advisory and guidance documents. Two commission services (ECVAM and DG Enterprise) and ICCVAM were part of a GLP working group to draft an Organisation for Economic Cooperation and Development (OECD) guidance document on GLP and *in vitro* toxicology that was finalized in May 2004.

ECVAM is also playing a leading role in drafting a new guidance document on GCCP. The aim of this GCCP document is to reduce uncertainty in the development and application of animal and human cell and tissue culture procedures and products, by encouraging greater international harmonization, rationalization and standardization of laboratory practices, quality control systems, safety procedures, recording, reporting, and compliance with regulations and ethical principles. To give this document an international dimension, ECVAM invited ICCVAM as part of the steering group who drafted this document (Coecke et al., 2005).

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66 Animal Models of Contact Urticaria

Antti I. Lauerma and Howard I. Maibach

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66.1 INTRODUCTION

Contact urticaria is an important skin disease. Products made from natural rubber latex have caused an increase in allergic (immunologic) contact urticaria, often also causing occupational disability. Other forms of contact urticaria are also rather common. The symptoms experienced in contact urticaria range from local itch to systemic anaphylaxis. The common factor in urticaria is the release of inflammatory mediators from cutaneous mast cells, such as histamine, cytokines, and chemokines, which cause pruritus and swelling of the skin tissue.

Contact urticaria presents as two different entities: immunologic contact urticaria (ICU) and nonimmunologic contact urticaria (NICU). They are separate in their mechanisms and etiology, and some differences in their clinical pictures may be seen. The most important distinguishing factor is the role of immunologic memory in these diseases. ICU occurs only in patients sensitized previously to the causative agent, whereas NICU does not require immunologic memory and may occur in any person. Owing to these differences, the diagnostic procedures in patients differ.

As an increasing number of chemicals are being used in skin medication and care, contact urticarias to new substances are a constant threat. To avoid such problems, predictive tests for exclusion of possibilities in new substances causing contact urticaria are needed. Moreover, as ICU and NICU are common skin problems, medications are also needed. To develop new medications for contact urticarias, models of NICU and ICU would be highly desirable. As of today, *in vitro* models for ICU and NICU are not available. Therefore, this chapter will concentrate on *in vivo* models, i.e., animal models.

66.2 MECHANISMS OF IMMUNOLOGIC CONTACT URTICARIA

Immunologic contact urticaria is mediated through IgE antibodies that identify molecules entering the body through skin and perceive them as foreign. IgE antibody-mediated reactions are most commonly seen in atopics. The molecule causing reactions has to penetrate epidermal layers, including the stratum corneum as well as basement membrane, before it attaches to IgE bound on mast cell surfaces in the dermis. The responsible molecules must have sufficient size and contain amino acid sequences to be able to bind to IgE. Therefore, the most usual molecules causing ICU are proteins or large-molecule-size polypeptides. Smaller peptides or chemicals have to bind to a carrier protein to be able to trigger immune response. After the responsible molecule binds to the IgE on mast cell, the cell releases inflammatory mediators, including histamine, cytokines, and chemokines, which cause itch, inflammation, and swelling in the skin. The swelling is seen as edema, the principal feature of urticaria [1].

66.3 RESPIRATORY CHEMICAL ALLERGY AS AN ANIMAL MODEL FOR IMMUNOLOGIC CONTACT URTICARIA

Anhydrides cause asthmatic-like symptoms in persons who have been exposed to them. The mechanisms related to trimellitic anhydride (TMA) have been well studied [2]. The immunologic reactions in experimental animals and patients feature anaphylactic (Type I), complement-mediated (Type II), antibody-complex-mediated (Type III), and cell-mediated (Type IV) reactions. Nonimmunologic (irritant) reactions

may also participate [3], possibly due to degradation of TMA to trimellitic acid.

TMA causes skin reactions if sensitization is done through skin contact [4]. The skin reactions appear in two phases, both immediate and delayed, implying that Type I and IV reactions are involved [1,4].

Sensitization of experimental animals can be done through airways [5] or alternatively through skin. Cutaneous sensitization can be done intradermally [3] or topically [4]. Cutaneous sensitization seems to induce both immediate and delayed skin reactions when a TMA-sensitized animal encounters the chemical next time.

Intradermal sensitization of guinea pigs can be done with TMA 30% in corn oil at 0.1 mL dose. The guinea pigs can be used for challenges 3–4 weeks after the injection.

BALB/C mice have been sensitized topically with TMA on shaven skin on the trunk that has been tape-stripped prior to application. The first dose has been 100 uL TMA at 500 mg/mL. To enhance development of anti-TMA-IgE-antibodies, a second sensitization has been performed with 50 uL TMA at 250 mg/mL at the same site. The animals have been used for elicitation 1 week after the second dosing.

In mice sensitized to TMA, a first immediate-type reaction due to reapplication is seen at 1 h after dosing and a second delayed-type swelling reaction is seen at 24 h. A dose-dependent swelling is also seen in nonsensitized animals [1], which can be caused by trimellitic acid, a hydrolization product of TMA [6]. Such reactions could possibly be a form of NICU.

Mice sensitized to topical TMA can be used for study of topical immunomodulating drugs. In one study, an antihistamine suppressed early, a glucocorticoid suppressed both early and delayed, and a nonsteroidal anti-inflammatory drug enhanced early skin reaction, in line with the clinical findings seen in patients in practice when these medications have been given in atopic IgE-mediated diseases [1].

Other haptens capable of inducing respiratory allergy, such as diphenyl-methane-4,4-diisocyanate (MDI) and phtalic anhydride are respiratory hapten allergens that possibly could also be used to establish an animal model for ICU [7,8].

66.4 CONTACT CHEMICAL ALLERGY AS AN ANIMAL MODEL FOR IMMUNOLOGIC CONTACT URTICARIA

When BALB/C mice are repeatedly sensitized for up to 48 h with strong contact allergen 2,4,6-trinitro-1-chlorobenzene (TNCB), an immediate-type reaction kinetic emerges at the expense of the more typical delayed-type response to this contact allergen. Such reaction kinetic shift coincided with an increase in the number of mast cells in the skin area used for sensitization and elicitation. Antigen-specific IgE was also seen, and the reactions were related to the increased number of mast cells on the site of application used [9].

66.5 PROTEIN ALLERGY AS AN ANIMAL MODEL FOR IMMUNOLOGIC CONTACT URTICARIA

Rabbits sensitized through airways or through skin with natural rubber latex show wheal-and-flare responses when prick tested [10]. Therefore, it could be that such animals can be used as an animal model for ICU to study its pathogenesis and possible medications to it. However, it has not been studied whether open application could be sufficient for ICU in this model as the rate of cutaneous penetration of natural rubber latex proteins has not been established. Also mice exposed to natural rubber latex have elevated IgE levels and eosinophilia [11]. Other proteins, such as ovalbumin, that are able to cause Type I IgE-mediated reactions [12], should be studied to see if they could be used in a similar manner.

66.6 NONIMMUNOLOGIC CONTACT URTICARIA: MECHANISMS

Nonimmunologic immediate contact reactions range from erythema to urticaria and occur in individuals who have not necessarily been previously exposed to them and who are also not sensitized to them. It is likely that NICU reactions are more common than ICU reactions. The reactions arise most likely from the causative agent's ability to induce release of histamine and leukotrienes from skin tissue, being therefore pharmacological in its nature. The agents causing NICU are numerous and include, among others, benzoic acid, sorbic acid, cinnamic aldehyde, and nicotinic acid esters. Provocative skin tests for NICU include the rub test and open test. The kinetics of NICU reactions are somewhat slower than those of ICU, i.e., the peak being at 45–60 min instead of 15–20 min [13].

66.7 ANIMAL MODELS FOR NONIMMUNOLOGIC CONTACT URTICARIA

Animal models have been searched in the hope to find a suitable screening method for compounds causing NICU [14]. The different agents causing NICU have often different mechanisms (the NICU reactions are pharmacological rather than immunological in etiology) and, therefore, an *in vivo* end point, i.e., thickness of ear pinnae, has been utilized.

Guinea pigs are more sensitive to NICU than mice and rats, and therefore guinea pigs have been used in most studies [15]. Substances studied are applied openly on guinea pig ear lobe, and edema is quantified with micrometer. The reactions are seen at their maximum after approximately 50 min after the application, the largest swellings being twofold. NICU model can also be used to study pharmacological agents to treat it [16].

66.8 CONCLUSIONS

It seems that mice sensitized to TMA and possibly also to other respiratory chemical allergens may be used as animal models for ICU. For NICU the guinea pig earlobe method may be most useful. These models need more refinement and standardization. Alternative methods may include *in vitro* mast cell or dendritic cell cultures with or without specific IgE obtained from patients for ICU; for NICU several tissue culture systems should be tried. Modern proteomics [17] and microarray [18] techniques are likely to yield alternative methods in time. If *in vitro* methods are used percutaneous absorption of the responsible agents should also be studied, as it is the prerequisite for both ICU and NICU.

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67 Diagnostic Tests in Dermatology: Patch and Photopatch Testing and Contact Urticaria

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67.1 INTRODUCTION

Diagnostic *in vivo* skin tests are used in dermatology to detect and define the possible exogenous chemical agent that causes a skin disorder, and hence are critical in their scientific documentation. These chemical agents often cause skin disorders by hypersensitivity mechanisms, which can thus be diagnosed by a provocative test (Lauerma and Maibach, 1995). The anatomical advantage of studying skin disorders is that the skin is the foremost frontier of the human body and, therefore, easily accessible for testing. Although it has been shown that differences in the reactivity of different skin sites exist, many causative agents may be tested locally on one skin site, thus exposing only limited areas of skin to the diagnostic procedures. Such procedures include patch, intradermal, prick, scratch, scratch-chamber, open, photo, photopatch, and provocative use tests. In cases of some generalized skin reactions, however, systemic exposure to the external agent may be necessary for diagnosis (Maibach, 2001).

The value of diagnostic tests is identification of the causative agent, which enables restarting of those chemicals or medications not responsible for the eruption. This chapter briefly describes the *in vivo* test methods used for making diagnoses of skin disorders. The skin disorders in which such tests are useful include drug eruptions, contact dermatitis and immediate contact reactions (contact urticaria), and possibly sensory (subjective) irritation (Table 67.1).

67.2 DRUG ERUPTIONS

Drug eruptions are a heterogeneous class of adverse skin reactions due to ingestion or injection of therapeutic drugs. The drug eruptions should ideally be diagnosed through systemic rechallenge, because many factors (e.g., systemic drug metabolism) may contribute to the process, and skin tests therefore are not as reliable. Because systemic challenge is not always easy to perform, skin tests may, however, precede such challenges, according to reaction type. If skin tests do not provide information about the causative agent and a medication needs to be restarted, the next step is a controlled drug rechallenge, preferably in a hospital environment (Kauppinen and Alanko, 1989).

The choice of provocation protocols depends on the type of reaction involved. Although much work has been directed toward classifying drug eruptions and elucidating their mechanisms, they are still not well understood. Many of them are presumably mediated by immunological mechanisms, but there are also nonimmunological drug eruptions, idiosyncrasies, in genetically predisposed persons. In cases of nonimmunological drug eruptions, skin tests are usually negative, and systemic provocations are also often negative (Tables 67.2 and 67.3). Immunological drug eruptions may be classified into the four reaction types according to Coombs and Gell (Bruynzeel and Ketal, 1989).

Anaphylactic (Coombs–Gell type I) reactions include anaphylaxis, urticaria, and angioneurotic edema. They are usually mediated by immunoglobulin E (IgE) antibodies. Penicillin is one well-known causative agent for type I reactions. Prick (Table 67.4) and scratch (Table 67.5) tests are used in diagnosis of type I reactions and are a relatively safe way of detecting the causative agent. Intradermal tests (Table 67.6) may also be used in such cases, although a much larger amount of the antigen is introduced into the body, which makes systemic reactions more likely. Also, *in vitro* tests such as the radioallergosorbent test (RAST) are used in diagnosis (Bruynzeel and Ketel, 1989). Because type I reactions are potentially

TABLE 67.1
Chemically Related Skin Disorders Diagnosable through Diagnostic Testing

Disorder	Mechanism[a]	Test Method
Drug eruption	Type I	Prick test or open test Scratch test Intradermal test Systemic challenge Patch test Systemic challenge Intradermal test Systemic challenge Patch test
	Type II Type III	Systemic challenge
	Type IV	Systemic challenge
	Nonimmunological	
Allergic contact dermatitis	Type IV	Patch test Intradermal test
		Open test or provocative use test (repeated open application test)
Contact urticaria syndrome (immediate contact reaction)	Type I	Open test (single application) Prick test Scratch test Scratch-chamber test
	Nonimmunological	Open test (single application)
Subjective irritation	Unknown	Lactic acid test Open test (single application)

[a] Types I–IV: Coombs–Gell classification of immunological mechanisms.

TABLE 67.2
Systemic Challenge: Protocol

The patient should be monitored under hospital conditions and emergency resuscitation equipment should be available throughout the study. Especially if the initial drug eruption was strong, challenge should be started at a low dose, that is, no more than one-tenth of the initial dose.

A dose of the suspected drug is given orally in the morning. The patient's skin, temperature, pulse, and other signs are followed at 1-h intervals for 10 h and recorded. If no reactions appear during 24 h, the challenge is repeated at a higher dose (e.g., one-third of the initial dose) the next morning.

If no reactions appear on days 1 and 2, then on the third morning a full therapeutic dose is given as a third challenge. If necessary, different drug challenges may be repeated every 24 h.

Note: See Kauppinen and Alanko (1989) for detailed instructions. The publication provides an unequaled clinical experience, and offers many valuable short-cuts in making scientifically based diagnoses.

TABLE 67.3
Systemic Challenge: Precautions

Challenge is not advisable if the patient has had: Anaphylaxis Toxic epidermal necrolysis (TEN) Stevens–Johnson syndrome or erythema multiforme systemic lupus erythematosus-like reaction

Extreme care should be exercised if the patient has had: Urticaria asthma any other immediate-type reaction, fixed drug eruption or its most severe form: generalized bullous fixed drug eruption (special variant of TEN)

Usually performed 1–2 months after the original eruption, except in severe reactions, when a longer interval (6 months–1 year) is advisable. Minimum provocative dose is generally less than one single therapeutic dose, except that in cases of severe bullous fixed drug eruption the initial test dose must be smaller (i.e., one-tenth to one-fourth of a single therapeutic dose).

Note: See Kauppinen and Alanko (1989) for detailed instructions.

life-threatening, systemic challenges (Tables 67.2 and 67.3) (Kauppinen and Alanko, 1989), if done, should be performed with extreme care, starting with very low doses, under hospital conditions. A physician should always be readily available, and the patient should be monitored frequently.

Cytotoxic (type II) reactions are mediated by cytotoxic mechanisms: quinine and quinidine are examples of causative agents. Patch tests (Table 67.7) may be attempted before systemic challenges (Tables 67.2 and 67.3) (Kauppinen and Alanko, 1989), for example, in the case of thrombocytopenic purpura caused by carbromal or bromisovalum (Bruynzeel and Ketel, 1989).

Immune complex-mediated (type II) reactions include Arthus and vasculitic reactions. Type III reactions are

TABLE 67.4
Prick Test

Materials:	(1) Allergens in vehicles. (2) Vehicle (negative control). (3) Histamine in 0.9% NaCl (positive control). (4) Prick lancets.
Method:	One drop of each test allergen, vehicle, and histamine control is applied to the volar aspects of forearms. The test site is pierced with a lancet to introduce the allergen into the skin.
Reading Time:	15–30 min
Interpretation:	An edematous reaction (wheal) of at least 3 mm in diameter and at least half the size of the histamine control is considered positive, in the absence of such reaction in the vehicle control.
Precautions:	General anaphylaxis not very likely, because of the small amount of allergen introduced, but a physician should always be available for such occurrences. The patient should not leave the premises during the first 30 min after the test.
Controls:	Required.

TABLE 67.5
Scratch Test

Materials:	(1) Allergens in vehicles. (2) Vehicle (negative control). (3) Histamine in 0.9% NaCl (positive control). (4) Needles.
Method:	One drop of each test allergen, vehicle, and histamine control is applied to the volar aspects of forearms or back, and needles are used to scratch the skin slightly at these sites.
Reading Time:	Up to 30 min
Interpretation:	Difficult because of unstandardized procedure. Edematous reaction at least as wide as the histamine control is considered positive in the absence of such reaction in the vehicle control.
Precautions:	As with prick test.
Controls:	Required.

mediated by immunoglobulins, complement, and the antigen itself, which form complexes. For example, sulfa preparations, pyrazolones, and hydantoin derivatives have caused vascular purpura via type III mechanisms. Intradermal tests (Table 67.6) may be tried for diagnosis before systemic challenges (Tables 67.2 and 67.3) (Bruynzeel and Ketel, 1989).

Delayed hypersensitivity (type IV) reactions are cell-mediated immune reactions involving the antigen, antigen-presenting cells, and T lymphocytes. Drug reactions of this type are often maculopapular or eczematous, although photoallergic reactions and fixed drug eruptions are also presumably mediated by type IV mechanisms. Other type IV reactions include some cases of erythroderma, exfoliative dermatitis, lichenoid and vesicobullous eruptions, erythema exudativum multiforme, and toxic epidermal necrolysis. Type IV reactions may be detected by patch tests with the

TABLE 67.6
Intradermal Test

Materials:	(1) Allergens in isotonic solution vehicles. (2) Solution vehicle (negative control). (3) Tuberculin (1 cc) syringes and needles.
Method:	0.05–0.1 mL of allergen solution and vehicle solution is applied intradermally to the skin of the volar aspects of forearms.
Reading Time:	30 min, 24 h, and 48 h
Interpretation:	Erythematous and edematous reaction at 30 min is suggestive of immediate type (type I) allergy in the absence of such a reaction in the vehicle control. Arthus reaction with polymorphonuclear leukocyte infiltration appearing in 2–4 h, which may progress into necrosis in hours or days, suggests cytotoxic (type III) reaction. Erythema and edema of at least 5 mm in diameter at 48 h indicates delayed-type hypersensitivity (type IV), for example, contact allergy.
Precautions:	The risk of general anaphylaxis is higher than in prick or scratch tests because of larger amount of allergen introduced; therefore, a physician should always be available for such occurrences. The risk is greater in asthmatic patients. The patient should not leave the premises during the first 30 min after the test.
Controls:	Required.

TABLE 67.7
Patch Test

Materials:	(1) Allergens in vehicle (e.g., petrolatum, ethanol, water). (2) Vehicles. (3) Aluminum chambers (Finn chamber), Scanpor tape, and filter papers (for solutions). Or ready-made patch test series (TRUE test).
Method:	Patches on tape or ready-made patch test series are applied on intact skin of the back. Filter papers are used for solutions: 17 μL of allergen in vehicle is used for each patch. Ready-made patch series is applied as is on similar skin sites. The patches are removed after 48 h.
Reading Time:	48 and 96 h
Interpretation:	Erythema and edema or more is positive. Distinguishing between allergic and irritant reaction is important. If the reaction spreads across the boundaries of the patch site, the reaction is more likely to be allergic, if the reaction peaks at 48 h and starts to fade rapidly after that, it may be irritant.
Precautions:	Intense skin reactions possible: these can be treated with topical glucocorticosteroids. Active sensitization possible.
Controls:	Required.

causative agent (Table 67.7) (Calkin and Maibach, 1993). In the case of a fixed drug eruption, in which the reaction reoccurs in the same skin site every time the drug is ingested, the patch test should be done in that particular skin site for a positive result (Alanko et al., 1987). For photosensitivity

TABLE 67.8
Photo Test

Materials:	Ultraviolet (UV) radiation source.
Method:	Minimal erythema dose (MED) of UVA or UVB is measured (1) while the subject is taking the suspected medication and (2) after discontinuing the same medication.
Interpretation:	If MED (UVA or UVB) is much lower while the subject is taking the medication, this suggests a photosensitive (phototoxic or photoallergic) reaction to the drug.

TABLE 67.9
Photopatch Test

Materials:	(1) Ultraviolet (UV) radiation source. (2) Patch test materials (see Table 67.8).
Method:	Two sets of patch test are applied for 48 h. After removal, one set is irradiated with UVA at a dose below MED (5–10 J/cm^2 or 50% of MED, whichever is smaller), and the other set is protected from UV dose.
Reading Time:	48 and 96 h
Interpretation:	Reaction only at irradiated site suggests photoallergy. Reaction at both sites suggests contact allergy. Reaction at both sites and a much stronger reaction at the irradiated site suggest both contact allergy and photoallergy.
Controls:	Required.

reactions, photo (Table 67.8) or photopatch tests (Table 67.9) should be done (Rosen, 1989). A negative patch test does not rule out the possibility that the tested drug may be causative. This is because patch testing involves potential limitations, such as insufficient penetration. In this case a systemic provocation (Tables 67.2 and 67.3) should be considered (Kauppinen and Alanko, 1989).

67.3 CONTACT DERMATITIS

Contact dermatitis is commonly divided into irritant contact dermatitis and allergic contact dermatitis. *Irritant contact dermatitis*, the more common of the two, is initiated by nonimmunological toxic mechanisms and is not diagnosed by patch testing, while allergic contact dermatitis is. In *allergic contact dermatitis* the patient becomes topically sensitized to a low molecular weight hapten and in subsequent topical contact develops an eczematous skin reaction, which is mediated by delayed hypersensitivity (type IV) mechanisms. Allergic contact dermatitis is diagnosed with patch tests (Table 67.7), intradermal tests (Table 67.6), or open tests (repeated application) (Table 67.10).

Of these three methods, patch testing is the most common and standardized. The problems involved in patch testing are insufficient penetration of the allergenic compound, which may result in false-negative results, and irritation from the test compound, which may cause a false-positive result. Also, patch testing may cause a worsening of eczema in other skin sites (excited skin syndrome) or active sensitization to patch compounds (Fischer and Maibach, 1990).

Two widely used methods for patch testing exist: the Finn chamber and TRUE test methods. Both have been shown to be reliable, especially when stronger reactions (contact allergies) are investigated (Ruhnek-Forsbeck et al., 1988). The TRUE test is somewhat easier to handle as it is ready to use. However, the Finn chamber method provides more flexibility for the dermatologist and the allergist to test substances not in routine patch test use. Regardless of the test method, the most important factor in successful patch testing is the experience and skill of the interpreter.

A standard patch test series is shown in Table 67.11. It is the standard series of the International Contact Dermatitis Research Group and the European Environmental and Contact Dermatitis Research Group (Andersen et al., 1991). A standard patch test series has been compiled to represent the most commonly encountered contact allergens, and it is meant to act as a screening tray. Its content is subject to change due to research findings about contact allergy (Andersen et al., 1991). A multitude of other patch test series are available when the causative agents of the individual patient's contact dermatitis are known better; these include, for example, patch test series for preservatives, rubber chemicals, topical drugs, and clothing chemicals. There are also special series to investigate occupational contact allergies in, for example, dental personnel or hairdressers (Wahlberg, 2003).

Patch tests should be applied on the back for 48 h and be read after removal. A second reading 24–48 h after patch removal is necessary, as irritant reactions, which are often easily misinterpreted as allergic, often tend to fade during the third and fourth day, while allergic reactions tend to persist. Additionally, with some allergens, such as corticosteroids and neomycin, late reactions often occur, possibly because of low percutaneous penetration. Therefore, a third reading approximately 1 week after patch application may be advisable, although this may be difficult to do routinely in practice (Lachapelle et al., 2003).

Intradermal testing (Table 67.6) has recently been shown to be of value in diagnosing hydrocortisone contact allergy (Wilkinsons et al., 1991); see Herbst et al. (1993) for a review of intradermal testing for allergic contact dermatitis. Open tests or repeated open application tests (Table 67.10) are not as sensitive as patch or intradermal tests, possibly because of insufficient penetration of the compound under unoccluded conditions (Hannuksela and Salo, 1986; Hannuksela, 1991).

67.4 CONTACT URTICARIA SYNDROME: IMMEDIATE CONTACT REACTIONS

Contact urticaria syndrome includes a group of skin reactions, that is, immediate contact reactions, which usually appear within 1 h of skin contact with the causative agent.

TABLE 67.10
Provocative Use Test (Open Test or Repeated Open Application Test)

Materials:	(1) Allergen in vehicle (petrolatum, ethanol, water) (2) Vehicle (3) Cotton-tipped applicators or other devices to spread the preparations
Method:	Patient applies allergen and vehicle on antecubital fossa (outpatient) or shoulder regions of upper back, twice a day, for 14 days or until a positive reaction appears.
Reading Time:	Patient reports if positive reaction appears. At day 7 and 14, the patient returns for reading of the test site.
Interpretation:	Erythema and edema or more is positive.
Precautions:	Active sensitization possible, but not yet documented.
Controls:	May be required.

TABLE 67.11
Standard Patch Test Series

Potassium dichromate	0.50% pet.
Neomycin sulfate	20.0% pet.
Thiuram mix	1.0% pet.
p-Phenylenediamine free base	1.0% pet.
Cobalt chloride	1.0% pet.
Benzocaine	5.0% pet.
Formaldehyde	1.0% aq.
Colophony	20.0% pet
Quinoline mix	6.0% pet
Balsam of Peru	25.0% pet.
PPD-black rubber mix	0.6% pet.
Wool alcohols (lanolin)	30.0% pet.
Mercapto mix	2.0% pet.
Epoxy resin	1.0% pet.
Paraben mix	15.0% pet.
p-tert-Butylphenol-formaldehyde resin	1.0% pet.
Fragrance mix	8.0% pet.
Ethylenediamine dihydrochloride	1.0% pet.
Quaternium 15	1.0% pet.
Nickel sulfate	5.0% pet.
Kathon CG	0.01% aq.
Mercaptobenzothiazole	2.0% pet.
Primin	0.01% pet.

Note: pet. = petrolatum vehicle; aq. = aqueous vehicle. Series is standard for the International Contact Dermatitis Research Group and the European Environmental and Contact Dermatitis Research Group.

TABLE 67.12
Scratch-Chamber Test

Materials:	(1) Scratch test materials (see Table 67.5). (2) Chambers.
Method:	As with scratch test, scratch sites are covered with aluminum chambers for 15 min.
Reading Time:	30 min
Interpretation:	See Table 67.5.
Precautions:	As with prick and scratch tests.
Controls:	Required.

TABLE 67.13
Open Test (Single Application) for Contact Urticaria Syndrome Immediate Contact Reactions

Materials:	(1) Allergen in vehicle (petrolatum, ethanol, water). (2) Vehicle. (3) Cotton-tipped applicators or other devices to spread the preparations.
Method:	Allergen and vehicle are applied to skin.
Reading Time:	Up to 1 h
Interpretation:	Urticarial reaction is positive.
Precautions:	See Table 67.4.
Controls:	Required to aid in disciminating immunological (ICU) from nonimmunological contact urticaria (NICU); in NICU, the reaction will be noted in most controls.

Immediate skin reactions are divided into immunological [immunoglobulin E (IgE) mediated] and nonimmunological immediate contact reactions. The symptoms range from mere itching and tingling to local wheal and flare. In cases of intense sensitivity, a generalized urticaria, systemic symptoms, and even anaphylaxis (contact urticaria syndrome) may occur (Lahti and Maibach, 1992).

Immunological immediate contact reactions are usually urticarial, although they may range from mere tingling in the skin to a generalized anaphylactic reaction in the whole body. Immunological immediate reactions are Coombs–Gell type I reactions mediated mainly via allergen-specific IgE bound to skin mast cells. Coupling of membrane-bound IgE by allergen causes mast cells to liberate histamine, which with other inflammatory mediators makes skin vessels permeable, and edema (urticaria) results. The sensitization in IgE-mediated contact urticaria may occur through skin or possibly the respiratory or gastrointestinal tract. Exposure through skin is the most likely route in occupational latex allergy in health personnel. The provocative *in vivo* methods usually performed first are prick test (Table 67.4), scratch test (Table 67.5), and

scratch-chamber test (Table 67.12). However, the test method simulating the clinical contact situation more realistically is the open application test (single application) (Table 67.13). A previously affected skin site is more sensitive to immunological skin reactions than a nonaffected site. In addition to *in vivo* methods, the diagnosis of immunological immediate contact reactions can be done with RAST, which detects antigen-specific lgE molecules from the patient's serum (Lahti and Maibach, 1992).

Nonimmunological immediate contact reactions range from erythema to urticaria and occur in persons not sensitized to the compounds (Lahti and Maibach, 1992). Nonimmunological contact reactions are probably more common than immunological contact reactions. They are possibly due to the causative agent's ability to release inflammatory mediators, such as histamine, prostaglandins, and leukotrienes from skin cells without the participation of IgE molecules. Agents capable of causing nonimmunological contact reactions are numerous: the most potent and best-studied agents are benzoic acid, sorbic acid, cinnamic aldehyde, and nicotinic acid esters. The test for diagnosis of NICU is the open application test (single application) (Table 67.13).

67.5 SUBJECTIVE IRRITATION

Although sensory (subjective) irritation is not fully characterized, there is evidence for a group of such persons, known as "stingers" (Maibach et al., 1989). The lactic acid test (Table 67.14) has been used experimentally to distinguish between "stingers," who more often have subjective irritation, and "nonstingers" (Lammintausta et al., 1988).

TABLE 67.14
Lactic Acid Test: Model for Sensory Irritation

Materials:	(1) Facial sauna. (2) 5% Lactic acid in water. (3) Vehicle (water). (4) Soap, paper towels, cotton-tipped applicators.
Method:	Facial area below eyes is cleansed with soap, paper towels, and water, rinsed with water, and patted dry. Face is exposed to sauna heat for 15 min. Moisture of face is blotted away. Lactic acid in water is rubbed on one side of face (cheek) and water on other. Face is exposed to sauna again.
Reading Time:	2 and 5 min after second sauna exposure.
Interpretation:	Any subjective sensation is graded by patient: 0 = none; 1 = slight; 2 = moderate; 3 severe. If cumulative score of two time points is 3 or more, patient is a "stinger."
Precautions:	Irritation may occur.

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68 Cosmetic Reactions

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68.1 INTRODUCTION

The term "cosmetic" is familiar, and its meaning has been expanded by an increase in the variety and complexity of substances used for cosmetic purposes. There are numerous ways to define and describe cosmetics. The Food, Drug, and Cosmetic Act, which the Food and Drug Administration (FDA) administers, defines cosmetics in the following manner (Code of Federal Regulations, 1986):

> The term "cosmetic" means[1] articles intended to be rubbed, poured, sprinkled, or sprayed on, introduced into, or otherwise applied to the human body or any part thereof for cleansing, beautifying, promoting attractiveness, or altering the appearance, and[2] articles intended for use as a component of any such articles: except the term shall not include soap.

Definitions pertaining to Europe and Japan are found in Barel,[3] Elsner,[4,5] and Baran.[6] Note two important aspects of this legal definition of cosmetics. First, in the United States, cosmetics in theory do not contain "active drug" entities of any type nor can they be promoted as altering any physiological state either in disease or health. Many countries do not recognize this legal distinction. The U.S. FDA classifies products into cosmetics, over-the-counter (OTC) drugs, and prescription drugs. By the U.S. definition, antiperspirants are OTC drugs regulated by the FDA through the OTC drug monograph system, while deodorants are cosmetics. The second aspect of the U.S. definition of cosmetics is the so-called soap exemption. Soap in the classic sense, as made of natural ingredients, is the type of soap that is exempted from the foregoing definition. However, if the soap product is made of detergent chemicals (synthetic surfactants), the product is regulated by the consumer product safety commission under the Federal Hazardous Substances Act (1960), as a household product. If the soap contains a therapeutic ingredient for a medical condition it is regulated as a prescription drug.[2] Likewise the classification of cosmetics is equally complex. The cosmetic industry itself divides the products into more general categories oriented as to their purpose as described in the definition.

Reactions to cosmetics constitute a small but significant portion of the cases of contact dermatitis seen by dermatologist in the United States. In a 5-year study, the North American Contact Dermatitis Group found that 5.4% of 13,216 patients tested were identified as having reactions caused by cosmetics.[7] This is an under representative of the true incidence because most patients who experience reactions to newly purchased cosmetics seldom consult a physician and just stop using the suspected cosmetic. In addition, they reported that 59% of the reactions caused by cosmetics occurred on the face including the periorbital area and 79% were females. Half of the cases later proven to evoke reactions to cosmetics were initially unsuspected. Reactions to cosmetics can have a variety of presentations, including subjective and objective irritations, allergic contact dermatitis, contact uriticaria, photosensitive reactions, pigmentation, and hair and nail changes.

68.2 CUTANEOUS REACTIONS

68.2.1 Irritant Dermatitis

68.2.1.1 Objective Irritation

Skin irritation has been described by exclusion as localized inflammation not mediated by either sensitized lymphocytes or by antibodies; for example, which develops by a process not involving the immune system. Skin irritation depends on endogenous and exogenous factors.[8] Predictive testing in human beings and rabbits can reliably detect strong or moderate irritants as ingredients in cosmetics or the products themselves. This allows manufacturers to test thoroughly to eliminate these potential hazards before marketing. There is recent evidence that no significant difference across skin types exists.[9] Because the stratum corneum of the facial skin is penetrated easily, more irritant reactions occur but always recognized clinically because of the complex biology of the human face. Many supposedly nonirritating moisturizers or emollient creams contain surfactants and emulsifiers that are mild irritants. These cosmetics are applied frequently to facial or inflamed skin resulting in irritant reactions. In product use testing, reproducing an irritant reaction may be difficult because penetrability of the stratum corneum varies with environmental conditions; and small panel testing may not account for the complexity and variance of the human genome. Provocative use testing may be performed at the original site of the reactions.

Application of some chemicals may directly destroy tissue, producing skin necrosis at the application site. Chemicals producing necrosis that results in the formation of scar tissue are described as corrosive. Chemicals may disrupt cell functions or trigger the release, formation, or activation of autocoids that produce local increases in blood flow, increase vascular permeability, attract white blood cells in the area, or directly damage cells. The additive effects of the mediators result in local skin inflammation. A number of, as yet, poorly defined pathways involving different processes of mediator generation appear to exist. Although no agent has yet met all the criteria to establish it as a mediator of skin irritation, histamine, 5-hydrotryptamine, prostaglandins, leukotrienes, kinins,[10] complement, reactive oxygen species, and products of white blood cells have been implicated as mediators of some irritant reactions.[11] Chemicals that produce inflammation as a result of a single exposure are termed acute or primary irritants.

Some chemicals do not produce acute irritation from a single exposure but may produce inflammation following repeated application, that is, cumulative irritation, to the same area of skin. Because of the possibility of skin contact during transport and use of many chemicals, regulatory agencies have mandated screening chemicals for the ability to produce skin corrosion and acute irritation. These studies are conducted in animals, using standardized protocols. However, the protocols specified by some agencies vary somewhat. It is not routinely appropriate to conduct screening studies for corrosion in humans, but acute irritation is sometimes evaluated

in humans after animal studies have been completed. Tests for predicting irritation in both animals and humans have been widely utilized. Predictive irritation assay in animals includes modified Draize test, repeated application patch tests, the guinea pig immersion test, and mouse ear test. Predictive human irritation assay includes many forms of the single application patch test, cumulative irritation assays, chamber scarification test, and exaggerated exposure test. These predictive assays have been reviewed.[12] Currently, *in vitro* skin corrosion test methods are being developed that avoid using animal models.[13] Sensitive bioengineering equipment used to evaluate pathophysiology of skin irritation includes transepidermal water loss,[14] dielectric characteristics, skin impedance, conductance, resistance, blood flow velocity, skin pH, O_2 resistance, and CO_2 effusion rate.[15] Several textbooks describe these methods in detail.[16–20] Also, irritant contact dermatitis can be avoided by prevention methods.[21]

68.2.1.2 Sensory or Subjective Irritation

Application of a cosmetic causing burning, stinging, or itching without detectable visible or microscopic changes, is designated as subjective irritation. This reaction is common in certain susceptible individuals occurring most frequently on the face. Some of the ingredients that cause this reaction are not generally considered irritants and will not cause abnormal responses in nonsusceptible individuals. Materials that produce subjective irritation include dimethyl sulfoxide, some benzoyl peroxide preparations, and the chemicals salicylic acid, propylene glycol, amyl-dimethyl-amino benzoic acid, and 2-ethoxy ethyl-methoxy cinnamate, which are ingredients of cosmetics and OTC drugs. Pyrethroids, a group of broad-spectrum insecticides, produce a similar condition that may lead to paraesthesia[22] at the nasolabial folds, cheeks, periorbital areas, and ears.

Only a portion of the human population seems to develop nonpyrethroid subjective irritation, and ethic variations in self-perceived sensitive skin has been noted.[23] Frosch and Kligman[24] found that they needed to prescreen subjects to identify "stingers" for conducting predictive assays. Only 20% of subjects exposed to 5% aqueous lactic acid in a hot, humid environment developed stinging response. All stingers in their series reported a history of adverse reactions to facial cosmetics, soaps, etc. A similar screening procedure by Lammintausta et al.[25] identified 18% of their subjects as stingers. Prior skin damage, for example, sunburn, pretreatment with surfactants, and tape stripping, increase the intensity of responses in stingers, and persons not normally experiencing a response report pain on exposure to lactic acid or other agents that produce subjective irritation.[26] Attempts to identify reactive subjects by association with other skin descriptors, for example, atopy, skin type, or skin dryness, have not yet been fruitful. However, Lammintausta et al.[25] showed that stingers develop stronger reactions to materials causing nonimmunologic contact urticaria and some increase in transepidermal water loss and blood flow following application of irritants via patches than those of nonstingers.

The mechanisms by which materials produce subjective irritation have not been extensively investigated. Pyrethroids directly act on the axon by interfering with the channel gating mechanism and impulse firing.[27] It has been suggested that agents causing subjective irritation act via a similar mechanism because no visible inflammation is present. An animal model was developed to rate paraesthesia to pyrethroids and may be useful for other agents.[22] Using this technique, it was possible to rank pyrethroids for their ability to produce paraesthesia. Lammintausta et al.[25] and Berardesca et al.[28] suggested that patients with subjective irritation have more responsive blood vessels. Assays and tests have been developed to quantify subjective irritation.[29,30]

As originally published, in human subjective irritation assay volunteers were seated in the chamber (110°F and 80% relative humidity) until a profuse facial sweating was observed.[24] Sweat was removed from the nasolabial fold and cheek; then a 5% aqueous solution of lactic acid was briskly rubbed over the area. Those who reported stinging for 3–5 min within the first 15 min were designated as stingers and were used for subsequent tests. Lammintausta et al.[25] used a 15-min treatment with a commercial facial sauna to produce facial sweating. The facial sauna technique is less stressful to both subjects and investigators and produces similar results.

68.3 ALLERGIC CONTACT DERMATITIS

Although allergic contact dermatitis is the most frequently diagnosed reaction to cosmetics and its incidence is rising,[31] it is clinically suspected initially in less than half the proven cases. Most cosmetics are a complex mixture containing perfumes, preservatives, stabilizers, lipids, alcohols, pigments etc. Frequently, these components are responsible for cosmetic allergy.[32] The clinical relevance of allergic contact dermatitis has been described.[33]

Allergic contact dermatitis is cell-mediated. This type of skin response is often referred to as delayed-type contact hypersensitivity because of the relatively long period (–24 h) required for the development of the inflammation following exposure. Lymphocytes are responsible for producing delayed-type hypersensitivity (DTH) and for regulation of the immune system. Lymphocytes leaving the lymphoid organs are "programmed" to recognize a specific chemical structure via a receptor molecule(s). If, during circulation through body tissues, a cell encounters the structure it is programmed to recognize, an immune response may be induced. To stimulate an immune response, a chemical must be presented to lymphocytes in an appropriate form.[34] Chemicals are usually haptens, which must conjugate with proteins in the skin or in other tissues in order to be recognized by the immune system. Haptens conjugate with proteins to form a number of different antigens that may stimulate an allergic response.[35] Hapten protein conjugates are processed by macrophages or other cells expressing proteins on their surface. Although the exact nature of this process is not completely understood, it is known that physical contact between macrophage and T cells

is required.[36] In the skin, keratinocytes produce interleukin,[37] an important regulatory protein for induction of DTH. Langerhans cells express Ia antigen and may act as antigen presenting cells.[38] Histologically, the DTH response has been described as a hyperproliferative epidermis with intracellular edema, spongiosis, intraepidermal vesiculation, and mononuclear cell infiltrate by 24 h. The dermis shows perivenous accumulation of lymphocytes, monocytes, and edema. No reaction occurs if the local vascular supply is interrupted and the appearance of epidermal changes follows the invasion of monocytes. The histology of the response varies somewhat by species.

Many factors modulate the development of DTH in experimental animals and humans. The method of skin exposure and rate of penetration influence the rate of sensitization. The effects of vehicle and occlusion are well documented.[39,40] Vehicle choice determines in part the absorption of the test material and can influence sensitization rate, ability to elicit response at challenge, and the irritation threshold. Application of haptens to irritated or tape stripped skin, the dose per unit area, repeated applications to the same site,[41] increased numbers of exposures (this applies through 10–15 exposures only), an interval of 2–6 days between exposures[41] and treatment with adjutant increase sensitization rates.[42] The development of DTH is under genetic control; all individuals do not have the capability to respond to a given hapten. In addition, the status of the immune system determines if an immune response can be induced. For example, young animals may become tolerant to a hapten, and pregnancy may suppress expression of allergy.[41] The intrinsic biological variables controlling sensitization can be influenced only by selection of animals likely to be capable of mounting an immune response to the hapten. Thresholds in contact sensitization using immunological models and experimental evidence have been described.[43] The extrinsic variables of dose, vehicle, route of exposure, adjuvant, etc., can be manipulated to develop sensitive predictive assays.

Appropriate execution of predictive sensitization assays is critical. All too often techniques are discredited when, in fact, the performance of the tests was inferior or study design, for example, choice of dose, was inappropriate. A common error in choosing an animal assay is using Freund's complete adjuvant (FCA) when setting dose–response relationships. The adjuvant provides such sensitivity that dose–effect relationships are muted. Although the dose must be high enough to ensure penetration, it must be below the irritation threshold at challenge to avoid misinterpretation of irritant inflammation as allergic. For instance, the quaternary ammonium compounds, for example, benzalkonium chloride, rarely sensitize but have been identified as allergens in some guinea pig assays. Knowing the irritation potential of compounds and choosing an appropriate experimental design will allow the investigator to design and execute these studies appropriately. John Draize developed the first practical animal assay to predict the proclivity of a chemical or a final product to produce allergic contact dermatitis. This test is widely used and forms the basis for current testing. Modifications to this test include the Buhler method, Freund's adjuvant, the FCA test, and the open epicutaneous test. An extensive review of these assays is found in Maibach and Marzulli.[12] If done properly, these tests will identify most of the contact allergens. Human testing supplements animal testing, but most sensitization studies have been done in animals.

The Draize repeated insult patch test is the standard human assay to identify the propensity of a chemical to induce ACD modifications to this test have been developed. A complete review of assays is found in Maibach and Marzulli.[12] Patch testing of patients with suspected cosmetic contact dermatitis is discussed later in this chapter, and the scientific basis of patch testing can be found in Ale and Maibach.[44] Recent prevention and treatment of allergic contact dermatitis has been discussed.[45]

68.4 CONTACT URTICARIA SYNDROME

Contact urticaria has been defined as a wheel-and-flare response that develops within 30–60 min after exposure of the skin to certain agents.[46] Symptoms of immediate contact reactions can be classified according to their morphology and severity. Itching, tingling, and burning with erythema is the weakest type of immediate contact reaction. Local wheel-and-flare with tingling and itching represents the prototype reaction of contact urticaria. Generalized urticaria after local contact is rare but can occur. Signs and symptoms in other organs can appear with the skin symptoms in cases of immunologic contact urticaria syndrome. This includes asthma, angioedema, and anaphylaxis.

The strength of the reactions may greatly vary, and often the whole range of local symptoms—from slight erythema to strong edema and erythema—can be seen from the same substance if different concentrations are used in skin tests.[47] Not only the concentration but also the site of the skin contact affects the reaction. A certain concentration of contact urticant may produce strong edema and erythema reactions on the skin of the back and face but only erythema on the volar surfaces of the lower arms or legs. In some cases, contact urticaria can be demonstrated only on damaged or previously eczematous skin. Some agents, such as formaldehyde, produce urticaria on healthy skin following repeated but not single applications to the skin. Differentiation between nonspecific irritant reactions and contact urticaria may be difficult. Strong irritants, for example, hydrochloric acid, lactic acid, cobalt chloride, formaldehyde, and phenol, can cause clear-cut immediate whealing if the concentration is high enough, but the reactions do not usually fade away within a few hours. Instead, they are followed by signs of irritation; erythema, scaling, or crusting that are seen 24 h later. Some substances have only urticant properties (e.g., benzoic acid, nicotinic acid esters). Diagnosis of immediate contact urticaria is based on a thorough history and skin testing with suspected substances. Skin tests for human diagnostic testing are summarized by Von Krogh and Maibach,[46] and patch testing and photopatch testing for contact urticaria has been described.[48] Because of the risk of systemic reactions, for

example, anaphylaxis, human diagnostic tests should only be performed by experienced personnel with facilities for resuscitation on hand. Contact urticaria has been divided into two main types nonimmunologic and immunologic on the basis of proposed pathophysiological mechanisms.[49] Recent reviews list agents suspected to cause each type of urticarial response.[50,51] Some common urticants are listed in Table 68.1. A flow sheet designed by von Krogh and Maibach[46] can be used to approach testing in suspected cases (Table 68.2).

68.4.1 Nonimmunologic Contact Urticaria

Nonimmunologic contact urticaria is the most common form and occurs without previous exposure in most individuals. The reaction remains localized and does not cause systemic symptoms or spread to become generalized urticaria. Typically, the strength of this type of contact urticaria reaction varies from sensory complains of sting, itch, or burn to an urticarial response, depending on the concentration, skin site, and substance. The mechanism of nonimmunologic contact urticaria has not been delineated, but a direct influence on dermal vessel walls or a non-antibody-mediated release of histamine prostaglandins, leukotrienes, substance P, or other inflammatory mediators represents possible mechanisms. Lahti and Maibach[50] suggested that nonimmunologic urticaria produced by different agents may involve different combinations of mediators. Common nonimmunological urticans can be inhibited by oral acetylsalicylic acid and indomethacin[52,53] and by topical dicloferac and naproxene gel, but not by hydroxyzine or terfenadine[54] and capsaicin. This suggests that prostaglandins and leukotrienes may play a role in the inflammatory response.

The most potent and best-studied substances producing nonimmunologic contact urticaria are benzoic acid, cinnamic acid, cinnamic aldehyde, and nicotinic esters. Under optimal conditions, more than half of a random sample of individuals show local edema and erythema reactions within 45 min of application of these substances if the concentration is high enough. Benzoic acid and sodium benzoate are used as preservatives for cosmetics and other topical preparations at concentrations from 0.1 to 0.2% and are capable of producing immediate contact reactions at the same concentrations.[55] Cinnamic aldehyde at a concentration of 0.01% may elicit an erythematous response associated with a burning or stinging feeling in the skin. Mouthwashes and chewing gums contain cinnamic aldehyde at concentrations high enough to produce a pleasant tingling sensation in the mouth and enhance the sale of the product. Higher concentrations produce lip swelling or typical contact urticaria in normal skin. Eugenol in the mixture inhibits contact sensitization to cinnamic aldehyde and inhibits nonimmunologic contact urticaria from this same substance. The mechanism of the putative quenching effect is not certain, but a competitive inhibition at the receptor level may be an explanation.[56] Provocative testing patients suspected of nonimmunologic urticaria with individual ingredients such as benzoic acid, sorbic acid, and sodium benzoate, common preservatives found in cosmetics, frequently will reproduce patient's symptoms.

TABLE 68.1
Some Agents Reported to Cause Urticaria in Humans

Immunologic mechanisms
Bacitracin
Ethyl and methyl parabens
Seafood (high molecular weight protein extracts)
Nonimmunologic mechanisms
Cinnamic aldehyde
Balsam of Peru
Benzoic acid
Ethyl aminobenzoate
Dimethyl sulfoxide
Unknown mechanisms
Epoxy resin
Lettuce/endive
Cassia oil
Formaldehyde
Ammonium persulfate
Neomycin

TABLE 68.2
Test Procedures for Evaluation of Immediate-Type Reactions in Recommended Order

1. Open application
Nonaffected normal skin
Negative
Slightly affected (or previously affected) skin
Negative Positive → positive diagnosis
2. Occlusive application (infrequently needed)
Nonaffected normal skin
Negative
Slightly affected (or previously affected) skin
Negative
3. Invasive (inhalant, prick, scratch, or intradermal injection)[a]

[a] When invasive methods are employed (especially scratch and inhalant testing) adequate controls are required.

68.4.2 Immunologic Contact Urticaria

Immunologic contact urticaria is an immediate type I allergic reaction in people previously sensitized to the causative agent.[46] It is more prevalent in atopic patients than in nonatopic pateints.[57] The molecules of a contact urticant react with specific IgE molecules attached to mast cell membranes. The cutaneous symptoms are elicited by vasoactive substances, that is, histamine and others, released from mast cells. The role of histamine is conspicuous, but other mediators of inflammation, for example, prostaglandins, leukotrienes, and kinins may influence the degree of response. Immunologic contact urticaria reaction can extend beyond the contact site, and generalized urticaria may be accompanied by other symptoms, for example, rhinitis, conjunctivitis, asthma, and even anaphylactic shock. The term "contact

urticaria syndrome" was therefore suggested by Maibach and Johnson.[49] The name generally has been accepted for a symptom complex in which local urticaria occurs at the contact site with symptoms in other parts of the skin or in target organs such as the nose and throat, lung, and gastrointestinal and cardiovascular systems. Anaphylactic reactions may result from substances that induce a strong hypersensitivity response or are easily absorbed from the skin.[58] Fortunately, the appearance of systemic symptoms is less common than the localized form, but it may be seen in cases of strong hypersensitivity or in a widespread exposure and abundant percutaneous absorption of an allergen. Foods are common causes of immunologic contact urticaria (Table 68.1). The orolaryngeal area is a site where immediate contact reactions are frequently provoked by food allergens, most often among atopic individuals. The actual antigens are proteins or protein complexes. As a proof of immediate hypersensitivity, specific IgE antibodies against the causative agent can typically be found in the patient's serum using the RAST technique and skin test for immediate allergy. In addition, the prick test can demonstrate immediate allergy. The passive transfer test (Prausnitz–Kustner test) also often gives a positive result. This is now performed in monkey rather than man.

68.5 ACNE AND COMEDONES

Acnegenesis and comedogenesis are distinct but often related types of adverse skin reactions to facial, hair and other products. Acnegenesis refers to the chemical irritation and inflammation of the follicular epithelium with resultant loose hyperkeratotic material within the follicule and inflammatory pustules and papules. Comedogenesis refers to the noninflammatory follicular response that leads to dense compact hyperkeratosis of the follicle. Mills and Berger[59] indicated that the time courses for the development of facial acne and comedones are different. While facial acne will appear in a matter of days, comedone formation in the human back and rabbit model takes longer to occur.

Classes of ingredients such as the lubricants isopropyl myristate and some analogs, lanolin and its derivatives, detergents, and D&C red dyes have been incriminated by the rabbit ear test.[60] Fulton[61] published a report about the comedogenicity and irritancy of commonly used cosmetics. Lists of comedogenic agents are not necessarily meaningful. Although they are important for pharmaceutical research and for the formulation of nonacnegenic products, they cannot alone predict the defects of the final product. The concentrations used in testing are often much greater than those in the final product. Thus, it is possible to use concentrations that are lower than the minimal acnegenic level. In addition, the vehicles in finished products can increase or decrease the acnegenic potential of individual compounds. In the final analysis, what is important is the testing of the finished product for its acnegenic and irritancy potential. Only when inadequate data are available, elimination regimen remains the only constructive approach to treat patients with suspected acne or comedones secondary to cosmetic use.

The rabbit ear assay is the major predictive animal model available. Kligman and Mills[62] developed the value of testing cosmetics and its ingredients by the rabbit ear assay. However, several improvements in the model have been proposed.[63] The test is not standardized. The AAD Invitational Symposium on Comedogeninity Panel (1989) suggested some guidelines for maximizing the usefulness of the rabbit ear model.

In 1972, Kligman and Mills[62] showed production of microcomedones on the backs of black men after testing cosmetics with occlusion. One test for evaluating comedogenicity in humans is the occlusive-patch application to the back followed by a cyanoacrylate follicular biopsy as described by Mills and Kligman.[64] The test material previously positive in the rabbit ear assay is applied for 4 weeks under occlusion to the upper portion of the back of people with large follicles. This test needs refinement. However, if this occlusive patch test is negative, it provides additional assurance that the test material may be nonacnegenic.

Bronaugh and Maibach[65] reported that results of the rabbit ear test correlate well with pustule formation noted in use tests of cosmetics performed on women's faces. They noted that some cosmetics may produce papulopustules after 3–7 days of use. The products were strongly positive in the rabbit ear model. This may represent a manifestation of primary irritancy. Correlative studies with rabbit pustulogenicity assay should be performed. The acute onset papulopustules are often described by the patient as a "breakout." The cause and effect relationship to cosmetics is strong but is often missed by the dermatologist. Jackson and Robillard[66] proposed the ordinary clinical-usage test. This test, conducted for 4–6 weeks, may not provide reliable information on comedogenesis. However, follicular inflammation may be noted within 1–2 weeks of applications done twice a day to the face of people with acne-prone oily skin.

It is not known how long the applications need to be continued to observe true comedogenesis. Clinical observations should be done at least weekly for the first 2 weeks of using the product to detect folliculitis. The acute papulopustular form will be identified in short-term testing (days, in contradistinction to months). However, to conclusively incriminate cosmetics as a cause of comedonal acne, long-term testing using a single cosmetic on the faces of women will have to be conducted and the disease produced. Lines of cosmetics will ideally be manufactured, which are screened with an appropriate rabbit ear test and then tested definitively in panels of acne-prone women for long term.

Wahlberg and Maibach[67] attempted to bridge the gap between comedo identified in the rabbit ear assay and the more common acute papulopustule by developing an animal model. The back of rabbits is pierced with a needle and dosed topically. The resulting lesion closely resembles that seen in man. Unfortunately, for several reasons including reluctance to performed animal testing, identification of acnegenicity premarketing remains a weak link in dermatotoxicology. The lesion occurs not only from ingredients of cosmetics but also from topical and systemic drugs.

68.6 PIGMENTATION

Hyperpigmentation of the face caused by contact dermatitis to ingredients in cosmetics occurs more frequently in dark complexioned individuals.[68] An epidemic of facial pigmentation reported in Japanese women was attributed to "coal-tar" dyes, principally, Sudan I, a contaminant of D&C Red No. 31.[69] The following fragrance ingredients have also been implicated—benzyl salicylate, ylang-ylang oil, cananga oil, jasmin absolute, hydroxycitronellal, methoxycitronellal, sandalwood oil, benzyl alcohol, cinnanuc alcohol, lavender oil, geraniol, and geranium oil.[70] Histologic examination shows hydropic degeneration of the basal layer, pigment incontinence, and little evidence of inflammation.[58] Mathias[71] reported pigmented cosmetic contact dermatitis due to contact allergy to chromium hydroxide used as a dye in toilet soap, and Maibach[72] reported hyperpigmentation in a black man sensitive to petrolatum. Dermatologists should search scrupulously for a causative agent in patients with hyperpigmentation. Eliminating the product frequently results in gradual fading of the pigment. Unfortunately, until a predictive assay is identified (14), most patients will be incorrectly identified as idiopathic.

Cosmetic chemicals have infrequently been associated with leukoderma. Nater and de Groot[73] and de Groot[74] listed chemicals associated with leukoderma. Recently, Taylor and colleagues[75] added seven more chemicals. Although Riley[76] reported that low concentrations of butylated hydroxyanisole were toxic in culture guinea pig melanocytes, Gellin et al.[77] could not induce depigmentation in guinea pigs or black mice by applying butylated hydroxyanisole. In addition, Maibach and colleagues[78] were unable to produce depigmentation after a 60-day occlusive application of hydroxytoluene to darkly pigmented men. The Cosmetic Ingredient Review Panel (1984) concluded that it is safe to use butylated hydroxyanisole (BHA) in the present practices of use.

Hydroquinone has produced depigmentaion in humans. Although it is a weak depigmenter, at 2% concentration, it is a stronger depigmenter at higher concentrations and with different vehicles. Hydroquinone, used as a bleaching agent, has caused postinflammatory hyperpigmentation in South African blacks. Findlay et al.[79] from South Africa reported a long-term complication of the use of hydroquinone-deposits of ochronotic pigment in the skin along with colloid milia. The melanocyte, despite intense hydroquinone use, escaped destruction and the site of the injury shifted to the dermis and the fibroblast. Polymeric pigment adhered to thickened, abnormal collagen bundles. In 1983, Cullison[80] reported that an American black woman developed this complication after intense use of a 2% hydroquinone cream. Prolonged use of hydroquinone followed by sun exposure may lead to exogenous ochronosis with colloid milium production. In addition, a few cases of persistent hypopigmentation have incriminated topical hydroquinone.[81] Pyrocatechol has structure and effects similar to hydroquinone. The most frequent use of hydroquinone and pyrocatechols is in rinse-off type hair dyes and colors in which the use is at 1% concentration or less. The Cosmetic Ingredient Review Panel declared hydroquinone and pyrocatechol safe for cosmetic use at 1% concentration or less. Monobenzyl ether of hydroquinone is a potent depigmenting agent and is not approved for cosmetic use in the United States. The only approved use for monobenzyl ether of hydroquinone is as a therapeutic agent for patients with vitiligo. *p*-Hydroxyanisole is a potent depigmenting agent in black guinea pigs at concentrations near to the ones used in cosmetics. It may cause depigmentation at distant sites from application in humans.

Angenilli and coworker[82] reported depigmentation of the lip margins from *p*-tertiary butyl phenol in a lip liner. The *p*-tertiary butyl phenol patch test site also depigmented and the presence of *p*-tertiary butyl phenol was confirmed by gas chromatography with mass spectroscopy. Most recently, Taylor and coworkers[75] reported four cases of chemical leukoderma associated with the application of semipermanent and permanent hair colors and rinses. They identified benzyl alcohol and paraphenylenediamine in three of the four cases. Depigmentation occurred at the hair color patch test sites in three of the four cases.

Mathias et al.[83] reported perioral leukoderma in a patient who used a cinnamic aldehyde-containing toothpaste. Wilkenson and Wilkin[84] reported that azelaic acid is a weak depigmenter and its esters do not depigment pigmented guinea pig skin.

68.7 PHOTOSENSITIVITY

Contact photosensitivity results from UV-induced excitation of a chemical applied to the skin. Contact photosensitivity is divided into phototoxic and photoallergic reactions. Phototoxic reactions may be experienced by any individual, provided that ultraviolet light contains the appropriate wavelengths to activate the compound and that the UV dose and the concentration of the photoreactive chemical are high enough. Clinically, it consists of erythema followed by hyperpigmentation and desquamation. Sunburn is the most common phototoxic reaction. However, photoallergic reactions require a period of sensitization. The reactions are usually delayed, manifesting days to weeks or years after the UV exposure. The major problem with photoallergic reactions is that the patient may develop persistent light reaction for many years after the chemical has been removed. These patients tend to be exquisitely sensitive to the sun and usually have very low UVB and UVA minimal erythema doses. With the exception of the epidemic caused by halogenated salicylanides in soap in the 1960s, photosensitivity accounts for a small number of cosmetic adverse reactions. Maibach and colleagues[85] reported only 9 of 713 patients with photoallergic and photosensitive reactions. Musk ambrette, a fragrance in some aftershaves, has been reported as a major cause of cosmetic photosensitivity reactions.

Predictive testing in human skin is not always definitive. In the 1960s, identification of TCSA and related phenolic compounds was accomplished by photopatch testing clinically involved patients. Subsequently, Willis and Kligman[86]

induced contact photoallergy to certain agents in normal human subjects using a modification of the maximization test, which was developed for evaluating the potential of chemicals to produce contact dermatitis.[87]

Kaidbey and Kligman[88] and Kaidbey[89] modified the photomaximization procedure. They were able to sensitize normal human volunteers readily to certain methylated coumarins derivatives, for example, TCSA, 3,5-DBS, chlorpromazine, and sodium omadine. A smaller number of positive induction responses was noted with TBS contaminated with 47% DBS, 4,5-DBS, Jadit, and bithionol. Negative results were obtained with para-arninobenzoic (PABA) and musk ambrette, which have produced photoallergic contact dermatitis clinically. To date there is no proven effective predictive testing model for photoallergic contact dermatitis in that most of the known photoallergens have been identified clinically and not in toxicologic assays. Furthermore, the refinement of risk assessment (not hazard identification) may be difficult, for example, sodium omadine is positive in the assay but not yet clinically in spite of extensive use.

68.7.1 Photopatch Testing

The criteria for separating allergic contact and allergic photocontact dermatitis utilizing patch-testing techniques are imprecise. General criteria and their interpretation are listed in Table 68.3. Often, the results are not all-or-none, as implied in the table. Frequently, there is a difference in response intensity, with either the contact or photocontact response being greater. All too infrequently serial dilutions are performed with either the putative antigen or the amount of ultraviolet light employed. Until a significant number of patients are so studied, it will be unclear how many of them represent contact versus photocontact sensitization.

Wennersten et al.[90] recommended that patients with suspected photocontact allergy be phototested before implementation of patch testing. The aim of this preliminary light testing is to detect any abnormal sensitivity to UVA and UVB wavebands. It is generally agreed that UVA sources are adequate and sufficient to elicit responses, an important convenience as UVA does not produce erythema in normal fair-skinned subjects until a dose of 20–30 J/cm^2 is delivered. High doses of UVA such as 10–15 J/cm^2 for photopatch testing are unnecessary. Such doses increase the possibility of adverse reactions and increase the incidence of phototoxic reactions. Despite widespread use, there is little standardization in the UVA dosage used in photopatch testing. Doses may range from 1–15 J/cm^2 at various centers.[91] The Scandinavian Photodermatitis Group was the first to formulate a protocol using 5 J/cm^2 of UVA in photosensitive patients, half their UVA MED for the procedure. Most photoallergies will be defined with a far smaller dose (e.g., 1 J/cm^2). Duguid et al.[92] showed that positive responses occur at 1.0, 1.0, and 0.7 J/cm^2 for Eusolex 8020, benzophenone-10 and benzophenone-3, respectively. In addition, Duguid and coworkers[22] confirmed the adequacy of 5 J/cm^2 or less as a photoelicitation dose. Although some data on the dose of light required to elicit a response existing, this remains incomplete and must be studied in context with the dose of antigen and the vehicle. Until the light and antigenic intensities are more fully defined, most physicians utilize a PUVA unit, a bank of UVA bulbs in a diagnostic unit or a hot quartz (Kromayer) unit, with an appropriate filter to remove any light with wavelengths below 320 nm (UVB). The effect of UV irradiation on photopatch test substances *in vitro* has been reported by Bruze et al.[93] It appears that 5 J of UVA is almost always adequate. All 5 J/cm^2 13 photoactive compounds formed photoproducts after UVA irradiation; 8 substances were decomposed by both UVA and UVB radiation; 5 by UVA alone. It is also possible that some patients may require UVB to elicit photoallergic dermatitis. However, since UVB testing is not done routinely, it may be some time before this is clarified. Epstein[94] observed that many patients are so sensitive to light that the dose delivered under an ordinary patch will elicit reactions. He provided details of testing the nonexposed site, utilizing a large light-impermeable black patch applied in a dimly lit room.

Commercial sources of appropriately diluted sunscreen antigens are not presently available in the United States. On request, many thoughtful manufacturers provide patch-test kits of individual ingredients for their products. A "standard" series of sunscreen antigens has been proposed by the International Contact Dermatitis Research Group (ICDRG). In Europe, these test kits are commercially available. These sunscreen antigens are available in 2% concentrations, although the maximum nonirritating doses of putative antigens in a given vehicle have not been defined. Maibach et al.[95] and de Groot[96] reported the test concentrations and vehicles for the dermatological testing of many cosmetic ingredients that may be in sunscreen formulations. We currently lack adequate virgin controls for the high concentrations used in contemporary formulations. When high concentrations are required to elicit allergic contact dermatitis, an impurity or a photoproduct may be the actual allergen.

The specific vehicle in which the allergens are dissolved or suspended is important.[97,98] The ICDRG list employs petrolatum as a diluent. This vehicle appears to be adequate to elicit reactions in many patients. It is clear, however, that the bioavailability of the antigen may be too limited in some cases. Thus, Mathias et al.[99] required ethanol to demonstrate PABA sensitivity, and Schauder and Ippen[100] noted more pronounced test reactions to avobenzone in isopropylmyristate

TABLE 68.3
Patch and Photopatch Testing

Contact Test Site Response	Photocontact Test Site Response	Interpretation
Positive	Positive	Allergic
Contact dermatitis		
Negative	Positive	
Photoallergic dermatitis		
Negative	Negative	Nonallergic
Sensitized		

TABLE 68.4
Strategy for Identifying the Cause When Routine Patch Testing is Negative

Intervention	Comment
Increase UVA dose	Avoid UVA erythema Use UVA control
Increase concentration of sunscreen	Upper limit of nonirritating dose not completely defined
Alter vehicle	Ethanol has been found to be effective
Test other components	Sunscreen manufacturers often helpful in providing test kits
Add suberythemogenic doses of UVB	
Perform provocative-use test on final formulation	
Consider "compound" allergy	

than petrolatum. This topic remains an area of investigation. Presumably each ingredient may require an optimal vehicle and concentration for eliciting a reaction.

Some patients develop dermatitis that appears allergic or photoallergic in a morphologic and historic sense, yet fails to demonstrate a positive patch or photopatch test, in spite of seemingly appropriate testing. Such false-negative reactions are more difficult to identify than false-positive reactions.[101] Table 68.4 provides the basic strategy employed in attempting to help these patients. Unfortunately, in some patients, even these extensive work-ups fail to elicit the etiology of their reactions.

Many of the reported positives test to date, and especially the cross-reaction studies, may well represent false-positives due to the excited skin syndrome. This state of skin hyperirritability often induced by a concomitant dermatitis is responsible for many nonreproducible patch tests. Bruynzeel and Maibach[102] detail strategies for minimizing such false-positives.

68.8 NAIL CHANGES

Paronychia, onycholysis,[103] nail destruction, and discoloration are some of the most common cosmetic adverse reactions found in the nails. The physician should obtain a detailed description of the nail grooming habits in patients who have paronychia, onycholysis, nail destruction, or nail discoloration because any of these problems may be caused by nail cosmetic usage. Nail discoloration has been reported with the use of hydroquinone bleaching creams and hair dyes containing henna.[104,105]

68.9 HAIR CHANGES

Permanents and hair straighteners are intended to break the disulfide bonds that give hair keratin its strength. Improper usage or incomplete neutralization of these cosmetics causes hair breakage. Hair that has been damaged by previous applications of permanent waves, straighteners, oxidation-type dyes, bleaches, or excessive exposure to sunlight and chlorine is more susceptible to this damage. The dermatologist should always take a complete history in these cases, including a detailed account of the use of drugs, to detect any causes of telogen or anagen effluvium. Careful examination of the hair shafts is essential to detect any preexisting abnormalities. Saving a sample of these hairs in the patient's record may be invaluable, should litigation against the beautician or supplier occur.[106]

68.10 INGREDIENT PATCH TESTING

The diagnosis and treatment of reactions to cosmetics has been facilitated by the FDA's regulation requiring the ingredient labeling of all retailed cosmetics.[107] The European community has also endorsed such labeling. The ingredients are listed in the order of descending concentration. Because of the complexity of the composition of fragrances, their compositions are not given but are listed simply as "fragrance." The regulation was designed to aid the consumer in identifying ingredients at the time of purchase; therefore, the list is often placed on the outer package, which may be discarded, rather than the container. Correspondence with the manufacturer or a trip to the cosmetic counter, however, can bring the needed information. This regulation, besides identifying ingredients, is helpful to dermatologists because it mandated a uniform nomenclature for cosmetic ingredients. The Cosmetic Toiletries and Fragrance Association (CTFA) *Ingredient Dictionary* published by the CTFA (1993) is the source for the official names. This dictionary provides a brief description of the chemical, alternative names, and names of suppliers: Without this key reference book, the dermatologist is at a distinct disadvantage in advising patients in this area.

The standard screening patch test tray includes some ingredients that are allergens found in cosmetics.[108] Imidazolidinyl urea, diazalidinyl urea, thimerosal, formaldehyde, and quaternium 15 are preservatives. Patch testing balsam of Peru screens for approximately 50% of the known allergic reactions to fragrance in the United States. Colophony and its constituents are used in the manufacture of eye cosmetics, transparent soap, and dentifrice.[109] If a patient has a positive patch-test reaction to one of these chemicals, clinician should consider allergic contact dermatitis to cosmetics a possible diagnosis. We emphasize that cosmetic contact dermatitis can often be unsuspected. A positive patch test should be interpreted cautiously because many cosmetics are mild irritants and excited skin state may cause false-positive results. Ideally, a positive patch test should be confirmed with a repeat test several weeks later or with a provocative-use test. Reassessment of the patient's history and presenting findings and patch testing with the patient's cosmetics may establish the diagnosis.

Once a product(s) has been implicated with patch testing, pin pointing the offending ingredient is an important part of the work-up so that the patient may spare recurring reactions. Cosmetic ingredient patch testing is complicated because

the proper concentration for closed patch testing is known only for a small percentage of these ingredients. Patch-test concentrations and vehicles has been proposed for less than 450 of the nearly 2000 cosmetic ingredients listed.[93] The texts by Nater and de Groot[73] and by Cronin[110] are important sources for clinicians seeking information on cosmetic ingredient patch testing.

Screening fragrance trays provide the most common fragrance allergens in the United States. Further information for patch-testing fragrance ingredients is reviewed in several articles.[111,112] When the clinical history, appearance of the reaction, or patch-test results lead the clinician to conclude that a cosmetic has caused an adverse reaction, it is important to obtain the ingredients for patch testing. The Cosmetic, Toiletry, and Fragrance Association publishes a pamphlet called "Cosmetic Industry On Call." This pamphlet lists the names of members of the industry who are willing to answer questions about their products. Physicians can contact these persons requesting specific ingredients for patch testing and information about patch-test concentrations. If the patient does or does not prove to have a reaction due to the cosmetics, the manufacturer should be notified of your results. Manufacturers will not always send materials for patch testing, and the patient cannot be treated successfully and counseled on how to avoid recurrences. Occasionally, "fractionated" samples will be sent for patch testing. Because irritant concentrations of ingredients may be present in these samples, they are often not suitable to use for closed patch testing. Some manufacturers will supply individual ingredients in the concentration that they appear in the product. These are often unsatisfactory for patch testing because the nonstandardized concentrations may be too low to provoke an allergic response or may be high enough to elicit irritation under occlusion.

68.11 COSMETIC PRODUCTS

68.11.1 Preservatives

After fragrances, preservatives are the next most common cause of cosmetic reactions.[7] The 10 most frequently used preservatives are listed in Table 68.5. A large number of specific studies have focused on individual preservatives as being identified as potential sensitizers and directly responsible for a number of adverse reactions.

Paraben esters (methyl, propyl, butyl, and ethyl) are nontoxic and nonirritating, preservatives that protect well against gram-positive bacteria and fungi, but poorly against several gram-negative bacteria including pseudomonads.[113] Parabens, the most widely used preservatives in topical products, have long been known to be contact allergens, when at relatively high concentrations and at a low frequency.[114] However, their potential for being the causative agents in cosmetic adverse reactions has not diminished their use, and in fact, it is on the increase. Fortunately, parabens compared to total use (tons *x* years) have a remarkable safety record. Although parabens may be sensitizers occasionally when applied to eczematous skin; cosmetics containing parabens infrequently cause clinical difficulties when they are applied to normal skin. Fisher[115] called this phenomenon the "paraben paradox." It is not known how often this phenomenon represents the excited skin state (due to high concentration of paraben in the patch-test mixture) rather than the paraben paradox.

TABLE 68.5
Preservative Frequency of Use (FDA Data)

Chemical Name	No. of Products Using Chemical
Methylparaben	6738
Propylparaben	5400
Propylene glycol	3922
Citric acid	2317
Imidazolidinyl urea	2312
Butylparaben	1669
Butylated hydroxyanisole (BHA)	1669
Butylated hydroxytoluene (BHT)	1610
Ethylparaben	1213
5-Chloro-2-methyl-4-isothiazolin-3-one (methylchloroisothiazolinone)	1042

Source: Adapted from *Cosmet. Toiletries*, 108, 97–98, 1993.

Imidazolidinyl urea (Germall 115) has low toxicity and is nonirritating. It has a broad antimicrobial activity especially when used in combination with parabens. Although formaldehyde is released on hydrolysis, the levels are too low to cause reactions in many formaldehyde-sensitive patients clinically or during patch testing. Diazolidinyl urea is a related preservative, whose use is increasing.

Quaternium-15 is active against bacteria but less active against yeast and molds. It is a formaldehyde releaser. A patient who has simultaneous positive patch-test readings to quaternium-15 and formaldehyde should be studied carefully, as this may require special instructions. The patient may be allergic to both ingredients or sensitive only to formaldehyde reacting to its release by this preservative in the occlusive patch test. In the latter situation, quaternium-15 may or may not be tolerated by the patient when present in cosmetics at 0.02–0.3% concentration. A product use test should clarify the situation. A negative test relates to the product tested and not to all products due to differences in bioavailability.

Formaldehyde as a preservative is used almost exclusively in wash-off products such as shampoos. Used in this manner, formaldehyde is seldom a cause of sensitization in the consumer and is only infrequently problematic for the beautician.[116,117]

Bronopol (2-bromo-2-nitropropane-1,3-diol) has a broad spectrum of activity, and most effective against bacteria. It is a formaldehyde releaser and may pose a problem for the formaldehyde-sensitive patient. In addition, this preservative may interact with amines or amides to produce nitrosamines or nitroamides: suspected carcinogens. Patch testing with standard concentrations may produce marginal irritation responses. Positives are best retested, and if positive followed by a provocative use test.

Benzoisothiazides have developed great popularity as preservatives. Kathon CG (5-chloro-2-methyl-4-isothiazohn-3-one and 2-methyl-4-isothiazolin-3-one) has been a preservative of choice by many formulators because of its broad and ease of formulation that is incorporated in many popular rinse-off products, and some leave on cosmetics under concentration restrictions. In spite of inducing sensitivity in guinea pigs at levels down to 25 ppm and elicitation levels down to 100 ppm and less, this preservative has infrequently produced sensitization from shampoo usage.[118] Methylchloroisothiazolinone and methylisothiazolinone have been the subject of several adverse reaction investigations. They have shown significant rates of sensitization at concentrations from 2 to 5%.[119–121] Diagnostic testing should be performed at 100 ppm in water because 300 ppm in water induces active sensitization (Bjorker and Fregert, personal communication). Possibly, 150–200 ppm might be more appropriate, but this requires additional study.

68.11.2 Preservation of the Future

The cosmetic industry experiences two major challenges: the elimination of all animal testing and the development of preservative-free cosmetics. In recent years, a strong socioeconomic pressure has focused interest and research on developing preservative-free products and preservation based upon natural extracts. Both of these approaches, although seemingly sound, scientifically and from a marketing position are fraught with problems. Natural preservatives are often complex mixtures with many unknown chemicals. It is likely that some of these active materials may present sensitization rates equal to or greater than that of synthetic materials. The Sixth Amendment of the EC Cosmetic Directive has called for the elimination of all animal testing of personal care products and ingredients by 2002, unless alternative methods cannot be developed by then. While most new raw materials will be able to use new, alternative safety testing methods, preservatives will experience difficulty in complying with existing requirements for chronic safety studies such as mutagenicity and teratogenicity, without relying on animals. Almost all regulatory agencies currently require the use of *in vivo* methods to confirm the safety of biocidal ingredients. *In vitro* test will most likely be used first as prescreening test. At the present time, this may reduce the need for some animal testing. However, it is unlikely that all *in vivo* methods of in the near future biocide safety will be replaced in the near future. Before *in vitro* assays can replace animal testing they should be validated against the known *in vivo* testing. This is an unfortunate drawback of increasing the safety testing cost since the cost of acute *in vitro* testing is as high as acute *in vivo*.

68.11.3 Emulsifiers

Creams and lotions require the presence of an emulsifier to allow the combination of water and five oil. Emulsifiers may act as mild irritants especially if applied to slightly damaged skin. Pugliese[122] suggested that increased epidermal cell renewal or "plumping" of the skin may be due to mild irritant effects of nonionic surfactants. Hannukesela et al.[123] patch tested over 1200 eczematous patients with common emulsifiers.

Another emulsifier, stearamidoethyl diethylamine posphate, has been implicated in four cases of cosmetic contact dermatitis.[124] Irritant reactions are seen at the same concentration as allergic responses. When 5% triethanolarnine stearate in petrolatum was tested, 9.5% of the patch tests showed irritant reactions. Even positive reactions to 1% triethanolamine in petrolatum should be confirmed by retesting and the provocative-use testing.

68.11.4 Lanolin

Lanolin is a mixture of esters and polyesters of high molecular weight alcohols and fatty acids. This naturally occurring wax varies in its composition depending on its source. Adams and Maibach[7] reported in the North American Contact Dermatitis Group (NACDG) study that lanolin and its derivatives remain among the ingredients that most commonly cause allergic contact dermatitis in cosmetics. Because of its superior emollient properties, lanolin is a popular ingredient for cosmetics.

Machovcova et al.[125] patch tested 12,058 patients, in the Czech Republic, who had been suspected for contact dermatitis. 63.5% of patients demonstrated at least one positive reaction, with lanolin alcohol being shown as a common allergen (3.0%). Patch testing is done most accurately using 30% wool alcohols in petrolatum. Kligman[126] tested 943 healthy young women with hydrous lanolin and 30% wool wax alcohol. The results were interpreted as follows: no positive allergic patch tests were read, but irritant reactions to wool alcohols were common. Clark et al.[127] estimated that the incidence of lanolin allergy in the general population is 5.5/million. Lanolin is an important sensitizer when it is applied to eczematous skin eruptions, especially stasis dermatitis. However, cosmetics containing lanolin applied to normal skin are generally harmless. Cronin[110] reported only 26 cases of lanolin–cosmetic dermatitis seen between 1966 and 1976 in women.

In all of these cases (26), the lanolin–cosmetic dermatitis affected the face at some time during its course; almost half showed eyelid involvement. The history was of intermittent eruptions often with swelling and edema.

68.11.5 Eye Makeup Preparations

Mascara, eyeliner, eye shadow, and eyebrow pencil, or powders are the most commonly used eye-area makeups. The upper eyelid dermatitis syndrome is complex and often frustrating to the patient and dermatologist, because of chronicity and failure to respond to our well-intentioned assistance. Causes that we have documented are included in Table 68.6.

Although patients often consider this as a reaction to eye makeup, the association is seldom proven. In the NACDG study, 12% of the cosmetic reactions occurred on the eyelid

TABLE 68.6
Some Causes of Upper-Eyelid Dermatitis Syndrome

Irritant dermatitis
Allergic contact dermatitis
Photoallergic contact dermatitis
Phototoxic dermatitis
Contact urticaria
Seborrheic dermatitis
Rosacea diathesis
Psoriasis
Collagen vascular diseases
Conjunctivitis
Blepharitis
Dysmorphobia

but only 4% of the reactions were attributed to eye makeup.[7] A workup of patients with eyelid dermatitis includes a careful history of all cosmetic usage, because facial, hair, and nail cosmetic reactions appear frequently on the eyelids.[128] Reactions to cosmetics on the eyelid are often the irritant type; and to further complicate their diagnosis, they may be due to cumulative irritancy—the summation of several, mild irritants (climatic, mechanical, or chemical).

When the history does not clearly incriminate certain cosmetics, test with the screening patch-test trays as well as all cosmetics that may reach the eye area directly or indirectly. Occlusive patch tests with eyeliner or mascara may give an irritant reaction, thus weakly positive results are interpreted cautiously. Waterproof mascaras must be dried thoroughly for 20 min to volatilize hydrocarbon solvents before occluding, and even with this precaution. Epstein noted some irritant reactions.[129] Positive patch tests are repeated for confirmation, and individual ingredient patch testing should be carried out whenever possible. Patch-testing cosmetics used in the eye area may occasionally give false-negative results when testing is done on the back or extremities.[128] A provocative use test performed in the antecubital fossa or the eyelid itself may ultimately prove the diagnosis.

In the United States, the pigments used in eye area cosmetics are restricted. No coal-tar derivatives may be incorporated; only purified natural colors or inorganic pigment or lakes of low allergic potential are used. Nickel contamination of iron oxide pigments has been implicated as a cause of allergic reaction to these cosmetics in the nickel-sensitive user.[130] Eye cosmetics are seldom fragranced, but other known allergens are used in these cosmetics. Almost all eye area cosmetics are preserved with parabens combined with a second preservative such as phenyl mercuric acetate, imidazolidinyl urea, quaternium-15, or potassium sorbate. The following antioxidants are sensitizers found in eye cosmetics: butylated hydroxytoluene, butylated hydroxyanisole,[131] propyl gallate,[132] ditert-butyl hydroquinone,[133] resins: colophony[132] and dihydroabietyl alcohol,[134] bismuth oxychloride,[135] and lanolin.[136] Propylene glycol may act as an irritant or sensitizer. Soap emulsifiers, surfactants, and solvents are all potential irritants used in these cosmetics. Allergic contact dermatitis from shellac,[137] prime yellow carnauba wax and coathylene,[138] and black iron oxide[139] found in mascara has been reported.

Infected corneal ulcers due to abrasions from mascara resulted when the mascaras were not properly preserved.[140] These preservation problems appear to be solved and reports of infected corneal ulcers have decreased. Patients should be urged to use their eye cosmetics hygienically and advised not to use eye cosmetics inside the lash line.

68.11.6 Hair Preparations (Noncoloring)

68.11.6.1 Permanents

Permanent waves are cosmetics that alter the disulfide bonds of hair keratin so that hair fiber configuration can be changed. The disulfide bonds of cystine are broken in the first step when the waving solution is applied to the hair wound around mandrels. In the second step, with neutralization, new disulfide bonds are formed by locking in the curl configuration of the hair.

The waving solutions contain reducing agents that can cause irritant reactions when allowed to run incautiously on the skin surrounding the scalp. Irritant reactions range from erythema to bullous dermatitis. Hair breakage and loss may result when permanent waves are used improperly—in too concentrated form, for too long time, or on hair previously damaged by dyes, straighteners, or permanent waves. Old-fashioned hot waves occasionally caused chemical burns, which scarred the scalp producing permanent alopecia, but modern permanents can cause breakage, which results in temporary loss.

In 1973, "acid permanents" were introduced for beauty salon use.[141] Acid permanents are the most widely used perm preparation today. These waving lotions, which contain anhydrous glyceryl monothioglycolate (GMT) in acid form, are mixed at the time of application with a water-based ammonium hydroxide solution to produce a neutral solution. The hair is covered with a plastic cap and placed under a hair dryer. Since the introduction of "acid perms," irritant and allergic reactions have been noted to occur on the hands of hairdressers and the face, neck, scalp, and hairline of their customers from use of these permanents.[142] Patch testing can be carried out with 1.0% glyceryl thioglycolate (GMT) in petrolatum or water (freshly prepared).

When clients are suspected of contact sensitization, GMT is one of the most likely sensitizers. Frosch et al.[143] stated that sensitization seems to be much less frequent in clients than in hairdressers due to less exposure; this was evident with ammonium persulfate (APS) (0% in clients versus 8% in hairdressers). Guerra et al.[144] studied 261 hairdresser's clients and reported similar results. They reported the mean frequencies of sensitization as follows: *p*-phenylenediamine (PPD) 7%, *o*-nitro-*p*-phenylenediamine (ONPPD) 5%, GMT 3%, ammonium thioglycolate (AMT) 1%, and APS 3%. Morrison and Storrs[145] indicated that the identification of GMT

sensitization in a patient is of particular importance, as the clinical symptoms may continue for months even if the use of acid permanent waves is stopped. The allergen clings to the hair and during shampooing, it is liberated in sufficient amounts to maintain the dermatitis. This may elicit dermatitis on the face and neck, which may be confused with allergy or irritancy to shampoo. The operational definition for allergic contact dermatitis has not been fulfilled. It is likely that most if not all patch-test reactions are irritant rather than allergic.

The "cold waves" contain thioglycolic acid combined with ammonia or another alkali to raise the pH. The concentration of the thioglycolic acid and alkali can be varied to change the products' speed of action or to suit the type of hair to be waved, that is, hard to wave, normal, or easy to wave. The neutralizer contains hydrogen peroxide or sodium bromate. These permanents have been alleged to rarely cause allergic reactions. Ammonium thioglycolate can be patch tested as 1 or 2.5% in petrolatum.[107] Positives should be confirmed with serial dilution patch testing and provocative-use testing. Another type of permanent used primarily at home is the sulfite wave. Although the sulfite wave produces less strong curls and is slower, the odor is more pleasant. Neutralization is done usually with bromates. Occasional allergic reactions have been alleged with these permanents. It is recommended to use 1% sodium bisulfite in water for patch testing.[146]

68.11.6.2 Straighteners

Straightening hair involves using a heated comb with petrolatum or a mixture of petrolatum, oils, and waxes. The petrolatum or "pressing oils" act as a heat-modifying conductor, which reduces friction when the comb travels down the hair fibers. Mechanical and heat damages can cause hair breakage. Over the years, the heated oils can injure the hair follicles leading to scarring alopecia.

Chemical straighteners containing sodium hydroxide, "lye," cleave the disulfide bonds of keratin thoroughly and straighten hair permanently. Experience and caution in applying these straighteners are important to avoid hair breakage and chemical burns. Similar products that contain guanidine carbonate mixed with calcium hydroxide are reputed to be milder. It is necessary to straighten new growth every several months. Care is taken not to "double process" the distal hair, which is already straightened. Some manufacturers advise against using permanent hair colors that require peroxide on chemically straightened hair to avoid damage. Sulfite straighteners, chemically similar to sulfite permanents, are best suited to relaxing curly Caucasian hair. "Soft Curls" have become a fashionable way of styling black hair. Ammonium thioglycolate and a bromate or peroxide neutralizer are used to achieve restructuring of the hair.

68.11.6.3 Shampoos

When shampoos are used, they have generally a short contact time with the scalp and are diluted and rinsed off quickly. These factors reduce their sensitizing potential. Complaints of consumers are commonly directed at their eye stinging and irritating qualities.[73] The importance of eye safety testing for these products became apparent 35 years ago, when shampoos based on blends of cationic and nonionic detergents caused blindness in some users.

Modern shampoos are detergent-based with a few containing small amounts of soap for conditioning. Anionic detergents and amphoteric detergents are occasional sensitizers.[147,148] Fatty acid amides used in shampoos as thickeners and foam stabilizers have caused allergic contact dermatitis in other products.[149] Formaldehyde or formaldehyde releasers may be used as a preservative in shampoos but formaldehyde rarely causes contact dermatitis in hairdressers or consumers related to the use of shampoos.[116,117] Other new preservatives used in shampoos include 5-cloro-2-methyl-4-isothozoline-3-one and 2-methyl-4-isothiazoline-3-one. Individual ingredient patch testing is necessary to incriminate allergens in shampoos. They produce false-negative results because they are diluted in the final product.

68.11.7 Hair-Coloring Preparations

Over 30 million Americans color their hair using five different types of dyes: Permanent hair dyes (type I) are mixtures of colorless aromatic compounds that act as primary intermediates and couplers. The primary intermediates, phenylenediamine (PPD), toluene-2,5-diamine (*p*-toluenediamine), and *p*-aminophenol are oxidized by couplers to form a variety of colors that blend to give the desired shade. These reactions take place inside the one hair shaft accounting for the fastness of these dyes. Permanent dyes are the most popular in the United States because of the variety of natural colors they can achieve. Semipermanent hair dyes (type II) contain low molecular weight nitrophenylenediamine and anthroquinone dyes, which penetrate the hair cortex to some extent. Their color lasts through approximately five shampoos. Temporary rinses (type III) are mixtures of mild, organic acids and certified dyes that coat the hair shaft. These rub and shampoo off easily. Vegetable dyes (type IV) in the United States contain henna, which only colors hair red. Metallic dyes (type V) contain lead acetate and sulfur. When they are combed through the hair daily they deposit insoluble lead oxides and sulfides that impart colors that range from yellowish-brown to dark gray.

Types I and II contain "coal-tar" hair dyes, and in the United States they must bear a label warning about adverse reactions. Instructions for open patch testing are given. The law requires patch testing be performed before each application of dye; in practice, this is seldom carried out in homes or salons. Coal-tar dyes are added occasionally to temporary rinses; these rinses must also bear a warning label and patch-test instructions.

A persistent and significant number of reactions to hair dyes are seen by dermatologists each year. Seven percent of the reactions to cosmetics diagnosed by the NACDG were caused by hair dyes[7]. Their severity ranges from mild

erythema at the hairline, ears to swelling of the eyelids and face, to an acute vesicular eruption in the scalp that requires prompt medical attention.

Most reactions to coal-tar dyes are reactions to PPD. Independent sensitization to toluene-2,5-diamine or 2-nitro-*p*-phenylenediamine dyes or resorcinol occurs rarely, but positive patch tests to toluene-2,5-diamine or 2-nitro-*p*-phenylenediamine dyes result generally from cross-sensitization to PPD.

One percent PPD in petrolatum is used in the standard closed patch test. Occasionally, patients who have a +1 reaction to PPD do not have a significant reaction when they dye their hair, but stronger patch-test results should warn patients not to use these hair dyes. *p*-Phenylenediamine is a colorless compound. Patch-test material gradually darkens as PPD oxidizes, and it should be stored in dark containers and should be made fresh at least yearly.

The products of PPD's oxidation are not allergenic. Reiss and Fisher[150] studied the allergenicity of dyed hair. Twenty patients sensitive to PPD were tested to freshly dyed hair in closed patch tests and all were negative. The findings of this study are important particularly to hairdressers, sensitive to PPD, who may work with dyed hair all day. Occasional case reports have appeared that suggested contact reactions occurred to another person's dyed hair.[151–153] Hillen et al.[154] found that only 1.5% of PPD patch tests caused late reactions. The authors further felt that of this 1.5%, most of the late reactions were due to patch-test sensitization. In light of this evidence, the German Contact Dermatitis Research group moved to update the German standard series to not include PPD 1% petrolatum.[154] We assume that the dyeing process must not have been carried out properly and that the unoxidized products remained on the hair.

Patients sensitive to PPD should be warned about possible cross-reactions with local anesthetics (procaine and benzocaine), sulfonamides, and *para*-aminobenzoic acid sunscreens. It is estimated that 25% of patients who are PPD-sensitive will react to semipermanent hair dyes. Patients who wish to try these as a substitute, should do an open patch test with the dye first.

Several patients have been reported who experienced immediate hypersensitivity reactions to PPD, and this spectrum of reactions to hair dyes should now be considered as a diagnostic possibility in appropriate patients.[155] Some patients complain of scalp irritation after dyeing their hair, but we are unware of published data that study the potential of these dyes for irritation. Some hair-dye reactions occur most prominently in light-exposed areas, but the phototoxic and photoallergic potential of coal-tar dyes has not been investigated. Severe reactions to hair dyes are uncommon, but not impossible, as even fatal anaphylactic reactions to hair dyes have been reported.[156]

Henna has not been reported to cause allergic contact dermatitis when used as a hair dye, but a case has been reported from coloring the skin with henna.[157] Cronin[158] described a hairdresser who noted wheezing and coryza when she handled henna; this patient had a positive prick test to henna. Edwards and Edwards[159] reported a case of contact dermatitis due to lead acetate in the metallic dyes.

When hair is bleached, ammonium persulfate is added to hydrogen peroxide to obtain the lightest shades. Ammonium persulfate has several industrial uses and it is known commonly to cause irritant reactions and allergic contact dermatitis occasionally. Methods of testing for immediate hypersensitivity include rubbing a saturated solution of ammonium persulfate on intact skin; scratch tests or intracutaneous tests using 1% aqueous solution of ammonium persulfate; and inhalation of 0.1 μg of ammonium persulfate powder. All of these methods can cause immediate hypersensitivity reactions including urticaria, facial edema, asthma, and syncope; thus, they should be performed only when emergency treatment for anaphylaxis is available. These reactions are histamine mediated, but it is not clear whether or not immunologic mechanisms are involved.[160] Hairdressers should be instructed that clients who develop hives, generalized itching, facial swelling, or asthma when the hair is bleached should not have the process repeated using persulfate. Clients experiencing such reactions should receive immediate medical attention.

68.11.8 Facial Makeup Preparations

Eleven percent of the reactions to cosmetics in the NACDG study were attributed facial makeup products, which include lipstick, rouge, makeup bases, and facial powder.[7] Prior to 1960, allergic reactions to lipsticks were common: most were caused by D&C Red 21 (eosin), an indelible dye used in longlasting, deeply colored lipsticks. The sensitizer in eosin proved to be a contaminant; improved methods of purification have reduced its sensitizing potential. Because eosin is strongly bound to keratin, patch tests are performed with 50% eosin in petrolatum.

Other dyes have occasionally been reported as sensitizers. Cronin[110] reported reactions to D&C Red 36, D&C Red 31, D&C Red 19, D&C Red 17, and D&C Yellow 11. The latter is a potent sensitizer, seldom used in lipsticks, but reported also as a sensitizing agent in eye cream[161] and rouge as well as lipstick.[162] D&C Red 17 not permitted in lipstick in the U.S. D&C Yellow 10, produced by the sulfonation of D&C Yellow 11, is not a potent sensitizer.[163] Other sensitizers reported in lipsticks include castor oil acting as a pigment solvent[164] antioxidants propyl gallate and monotertiarybutylhydroquinone,[165] sunscreens phenyl salicylate and amyldimethyl aminobenzoic acid,[166,167] lanolin,[136] and fragrance.

Although reactions to lipstick are uncommon, dermatologists should consider this diagnosis even when the eruption has spread beyond the lips, because the sensitizing chemical may be present in cosmetics other than the lipstick. Do not neglect to test each lipstick that the patient uses performing closed as well as photopatch tests, because some of the dyes used may be photoallergens.

Rouge or "blush" is manufactured in various forms—powder, cream, liquid, stick, or gel. It is designed to highlight the cheeks with color. The composition is not unique: powders are similar to face powder, and creams and liquids are similar to foundation. To achieve bright shades, organic colors

are added to rouges as they are to lipsticks. D&C Yellow 11 caused allergic reactions to rouges as well as lipsticks.[138] Some women may use lipstick to color their cheeks in place of rouge, or rouge may be used all over the face to achieve a healthy glow. These practices need to be taken into account when evaluating patterns of contact dermatitis on the face.

Facial makeups or foundations are applied to the skin to give an appearance of uniform color and texture and to disguise blemishes or imperfections. They are produced in a variety of forms—emulsions of water and oil, oil-free lotions, anhydrous sticks, poured powders, and pancake makeups; and the amount of coverage given is determined by the titanium dioxide (TiO_2) content. Because TiO_2 reflects light, some ordinary makeups achieve sun protection factor (SPF) values of 2 or even 4.[168] In recent years, sunscreening agents have been added to some foundations to increase these SPF values. When sunscreening claims are made for these cosmetics, the U.S. government will consider these products as OTC drugs also. PABA derivatives, fragrances, emulsifiers, preservatives, propylene glycol, and lanolin are chemicals with significant sensitizing potential used in these makeups. Synthetic esters, such as isopropyl myristate and lanolin derivatives added to these makeups, have been implicated as causes of acne by the rabbit's ear test.[62] However, most cosmetic reactions, such as contact allergy, are due to fragrance mixtures and formaldehyde.[169]

Calnan[170] described a woman who had a positive patch test to her foundation on two occasions, and her facial eruption flared when she used this foundation. However, patch testing the individual ingredients of this foundation was negative. Calnan raised the possibility of compound allergy—the allergen is produced by a combination of more than one ingredient. Despite some noted adverse reactions to cosmetics, facial cosmetics are considered safe consumer products.[171]

68.11.9 Sunscreen

Sunscreens can be classified into two major types: chemical and physical.[172] Physical sunscreens such as titanium dioxide and zinc oxide reduce the amount of light penetrating the skin by creating a physical barrier that reflects, scatters, or physically blocks the ultraviolet light reaching the skin surface. Chemical sunscreens, however, reduce the amount of light reaching the stratum corneum by absorbing the radiation. Examples of chemical sunscreens include PABA and its derivatives such as padimate O, cinnamates, benzophenones, salicylate derivatives, and dibenzoylmethane derivatives.

Because chemical sunscreens are applied topically to the skin in relatively high concentrations (up to 26%), contact sensitization can occur.[173,174] Similarly, because these chemicals absorb radiation, they have the potential to cause photosensitization. Both types of sensitization can occur with not only the various sunscreening agents but also with excipients such as emulsifiers, antioxidants, and preservatives that are included in the various hydroalcoholic lotions, ointments, oil-in-water, or water-in-oil emulsions. Despite extensive sunscreen use, there have been infrequent published reports of sunscreen-induced side effects, including allergic/photoallergic reactions, but we have inadequate data to accurately predict the degree of hypersensitivity to sunscreening agents due to the lack of a well-developed adverse reaction reporting system.

Numerous sunscreening preparations are currently sold in the United States. Table 68.7 lists examples of the sunscreen formulations sold in the United States together with the active ingredients and their SPF, a measure of sunscreen protection against sunburn. Most of the formulations that are combination sunscreens contain one or more UVB absorbers and a UVA absorber to provide much needed protection against the damaging effects of UVA. Several sunscreens also include physical blockers such as titanium dioxide.

In general, as the SPF of the sunscreen formulation increases, the number of active ingredients increases to three or four, and in some cases the total amount of active sunscreens increases up to 26%. Not all formulations list the specific concentrations of the active ingredients, and it is possible that some may contain higher concentrations. As with many chemicals, increasing the concentrations of the active ingredients may increase the likelihood of sensitization.[175] The majority of sunscreens contain octyl dimethyl PABA (padimate O) as the main UVB absorber. The cinnamate derivative, octyl methoxycinnamate (parsol MCX), is also used as a UVB absorber.

Most sunscreens with more than one ingredient contain oxybenzone as the additional ingredient. The absorption peak of this compound lies in the UVB region and extends partially into the UVA. Other agents that absorb in the UVA region include sulisobenzone, dioxybenzone, menthyl anthranilate, and avobenzone. The latter chemical, which has an absorption maximum in the middle of the UVA region (358 nm), has been approved in the United States in two sunscreen formulations (Photoplex, Herbert Laboratories, Santa Ana, California and Coppertone, Sun and Shade) (Table 68.7).

Published reports of contact and photocontact sensitization and contact urticaria induced by sunscreening agents are listed in Table 68.1. Representatives of all major sunscreen categories including PABA derivatives, anthranilates, salicylates, cinnamates, and benzophenones that have caused allergic reactions are described as follows:

PABA. In 1976, Willis[176] suggested that the sensitization potential of *p*-amino benzoic acid was minimal. Wennersten et al.[177] reported that a total of 73/1883 (3.9%) subjects tested with 5% PABA in alcohol in the Scandinavian standard photopatch tray had either allergic or photoallergic responses to PABA. These subjects represent 73% of the total number of subjects with contact and photocontact sensitization to PABA (Table 68.1). The use of PABA as a sunscreening agent in Europe and the United States has decreased significantly in recent years. PABA has been replaced by ester derivatives such as padimate O that, unlike PABA, are not water soluble and tend

TABLE 68.7
Selected Sunscreen Formulations Available in the United States

Trade Name	SPF	Active Ingredients
Four sunscreening ingredients		
Coppertone (Plough)	30	Padimate O, Parsol MCX, octyl salicylate, oxybenzone
Sundown (Johnson & Johnson)	30	Parsol MCX, octyl salicylate, oxybenzone, titanium dioxide
	20	Padimate O, Parsol MCX, octyl salicylate, oxybenzone
Cancer Garde (Eclipse Labs)	30	Padimate O, Parsol MCX, oxybenzone, titanium dioxide
T/I Screen (T/I Pharmaceuticals)	30+	Parsol MCX, octocrylene, octyl salicylate, oxybenzone
Block Out (Carter Products)	30	Parsol MCX, padimate O, octyl salicylate, oxybenzone
Supershade (Plough)	44	Parsol MCX, padimate O, homosalate, oxybenzone
Three sunscreening ingredients		
Solbar (Person and Covey)	50	Parsol MCX, octocrylene, oxybenzone
PreSun for Kids (Westwood)	39	Parsol MCX, octyl salicylate, oxybenzone
PreSun 29	29	Parsol MCX, octyl salicylate, oxybenzone
Bain de Soled (Bain de Soled)	30	Padimate O, Parsol MCX, oxybenzone
Ultrashade (Plough)	23	Padimate O, Parsol MCX, oxybenzone
Total Eclipse (Eclipse Labs)	15	Padimate O, octyl salicylate, oxybenzone
Sundown (Johnson & Johnson)	15	Padimate O, Parsol MCX, oxybenzone
Two sunscreening ingredients		
Supershade (Plough)	8, 15	Parsol MCX, oxybenzone
Coppertone (Plough)	4, 6, 8, 15	Padimate O, oxybenzone
Shade (Plough)	4, 6	Padimate O, oxybenzone
PreSun (Westwood)	8, 15	Padimate O, oxybenzone
Water Babies (Plough)	15	Parsol MCX, oxybenzone
Sundown (Johnson & Johnson)	4, 6, 8	Padimate O, oxybenzone
Block Out (Carter Products)	15	Padimate O, oxybenzone
Photoplex (Herbert Labs)	15	Padimate O, avobenzone
One sunscreening ingredient		
Coppertone (Plough)	2	Octyl salicylate
Bain de Soleil (Bain de Soleil)	2, 4	Padimate O
Eclipse (Eclipse Labs)	5	Padimate O
	10	Glyceryl PABA

to remain on the surface layer with less than 10% penetrating the corneum even after 24 h.[178] These PABA esters appear to be less sensitizing than PABA; however, there is no data to substantiate this impression.

PABA derivatives. Sensitization to glyceryl PABA have been reported for the last 30 years.[179,180] Many of the cases of glyceryl PABA sensitization showed uniform strong reactions to benzocaine, suggesting that the sensitization may be due to the presence of impurities in the glyceryl PABA. This suggestion was first made by Fisher[181] and has since been confirmed.[182] Benzocaine impurities (1–18%) occurred in many commercial sources of glyceryl PABA. Thus many of the early reports of contact allergy to glyceryl PABA may have falsely implicated glyceryl PABA as the sensitizer. Thune[183] reported two cases of allergic/photoallergic reactions to glyceryl PABA in which there was no reaction to benzocaine, suggesting true allergy to the PABA derivative. However, no allergic responses were observed when these subjects were patched with glyceryl PABA, which had been purified via high-pressure liquid chromatography.[184] This suggests the presence of an, as yet, unknown impurity (or impurities) other than benzocaine as the sensitization source. This shows the importance of utilizing purified raw materials in the manufacture of consumer products and the need for careful interpretation of patch test results.

Other PABA derivatives that have caused sensitization/photocontact sensitization include octyl dimethyl PABA (padimate O), amyl dimethyl PABA (padimate A), and ethyl dihydroxy PABA. The number of case reports of sensitization/photocontact sensitization with padimate O is less than that reported with PABA and glyceryl PABA suggesting a lower sensitization potential with this derivative. This may be because padimate O is not a true PABA ester since it does not contain the NH2 grouping present in glyceryl PABA, PABA, and benzocaine.[185]

Although padimate A was included[172] as a safe and effective sunscreening agent, this derivative can cause phototoxicity and may have accounted for the erythemal response observed by Katz[186]

30 min after sun exposure. This compound is no longer used in sunscreens in the United States. In addition to benzocaine impurities in the glyceryl PABA raw materials, some PABA esters contain 0.2–4.5% PABA.[187] It is possible that PABA impurities may account for some of the reports of sensitization to the PABA derivatives.

Salicylates. There are two cases of contact allergy and two reports of photocontact allergy to homomenthyl salicylate in the literature[188] and no reports of sensitization to octyl salicylate, the major salicylate derivative in many sunscreens.

Cinnamates. Cinnamates are chemically related to or are found in balsam of Peru, balsam of Tolu, coca leaves, cinnamic acid, cinnamicaldehyde, and cirmamon oil, ingredients used in perfumes, topical medications, cosmetics, and flavoring. Thune[183] reported eight cases of sensitivity to cinnamates, two cases of photoallergy to 2-ethoxyethyl-*p*-cinnamate, and six subjects with contact allergy to other cinnamates such as amyl cinnamaldehyde, amyl cinnamic acid, and cinnamon oil. Calnan[189] reported cross-sensitization among cinnamon derivatives.

Benzophenones. There have been reported cases of photocontact allergy[183] and contact allergy[190] to oxybenzone; and reports of contact allergy[7] and photocontact allergy to sulisobenzone. Benzophenone-10 (mexenone), a benzophenone derivative not used in sunscreens in the United States, can also cause contact and photocontact dermatitis.[191,192]

Dibenzoylmethanes. Dibenzoylmethane derivatives such as isopropyldibenzoylmethane (Eusolex 8020) and butyl dibenzoylmethane (avobenzone) have been incorporated in European sunscreens as UVA absorbers since 1980. Instances of contact allergy/photoallergy to sunscreens and lipsticks containing dibenzoylmethanes or these derivatives have been reported, although the majority of reports have been associated with the isopropyl derivative.[193–195] As a result, manufacturers stopped incorporating Eusolex 8020 into their products.[196,197] Recently, the manufacturers of Eusolex 8020 withdrew from the market.

There have been fewer reports of contact allergy/photoallergy to the butyl dibenzoylmethane derivative, avobenzone. It is possible that some of these reactions to avobenzone may have been cross-reactions resulting from prior exposure to the isopropyl derivative.[169] Greater utilization of these compounds with appropriate testing should help clarify their relative sensitization potential.

Camphor derivatives. 3-(4-Methyl-benzylidene) camphor (Eusolex 6300) is a sunscreening agent used extensively in Europe, often in combination with Eusolex 8020, but it is not approved for use in the United States. There have been several reports of allergic and photoallergic reactions to sunscreens containing this agent.[172]

Miscellaneous. Other chemical sunscreens that have caused allergic reactions include diagalloyl trioleate,[198] the glycerol ester of *o*-aminometa (2,3 dihydroxyproxy) benzoic acid,[199] a dioxane derivative;[200] and 2-phenyls-methyl-benzoazol (witisol).[201] None of these ingredients are approved for use in sunscreens in the United States.

Titanium dioxide. Physical blockers such as titanium dioxide and zinc oxide have the advantage of not being sensitizers, but may be so occlusive that they can cause miliaria.[202] Kaminester[203] reported that the inclusion of titanium dioxide in a PABA sunscreen blocked the appearance of photoallergy. It is possible that the reflection and scattering of light by titanium dioxide reduced the amount of UV light that penetrated the skin and elicited photo allergy.

Excipients. Contact allergy can also be caused by excipients included in sunscreens. These chemicals include mineral oil, petrolatum, isopropyl esters, lanolin derivatives, aliphatic alcohols, triglycerides, fatty acids, waxes, propylene glycol, emulsifiers, thickeners, preservatives, and fragrances. An extensive list of vehicle constituents in cosmetics that can cause allergic responses has been published.[73] De Groot et al.[74] indicated that preservatives, fragrances, and emulsifiers are the main classes of ingredients responsible for cosmetic allergy, with Kathon CG producing contact allergic reactions in 27.7% of subjects tested. Sunscreens available in the United States provide a complete list of ingredients including the excipients. The listing of all ingredients in sunscreens should be encouraged so that consumers, especially those with known sensitivities to chemicals, are fully informed about the composition of the formulation prior to the purchase and application of the product to the skin.

68.11.10 Manicuring Preparations

In the past, adverse reactions have been reported to numerous nail cosmetics that have been removed subsequently from the market because of reported hazards. Nail hardeners containing formaldehyde are in this category. In the United States, this type of hardener is permitted or used only on the free edge of the nail when the skin is protected from contact with the hardener. Some manufacturers sell products called hardeners but they have merely increased the resin content of ordinary nail enamel. Nail enamels including base coats and topcoats have a similar composition. The concentration of each of these chemicals depends on the quality to be achieved in the final product. The base coat will have increased amounts of resin to improve adhesion to the nail plate, but the topcoat has increased nitrocellulose and plasticizers to enhance gloss and abrasion resistance.

Toluene sulfonamide/formaldehyde resin (TSFR) is responsible for contact dermatitis not only around the nails

but also at sites distant from the fingers, commonly eyelids, around the mouth and chin, sides of the neck, on the genitalia, and rarely a generalized eruption. In contrast, free formaldehyde (FF) in nail hardeners cause mostly local reactions. Cronin[110] recommends 10% toluene sulfonamide/formaldehyde in petrolatum to perform a closed patch test. Nortaon[204] reported that FF hardeners are the most common cause of nail cosmetic reaction followed by methacrylate and cyanoacrylate resins, TSFR, acetone removers, and sodium and potassium hydroxide removers. Norton also reported onychomycosis, chromonychia, anonychia, and pterigum inversum unguis, secondary to FF nail hardeners. A small amount (0.1–0.5%) of FF is in the resin.[170] Fisher[205] proposed that patients who are allergic to this resin may wear nail polish without problems if they allow it to dry thoroughly with their hands quietly at rest. Those who find this inconvenient can be advised to purchase certain "hypoallergenic" brands of nail polish, which substitute alkyd or other resins. Ask the patient to check the ingredient list for toluene sulfonamide/formaldehyde resin as a precaution. The durability and abrasion resistance of these other resins is said to be inferior to toluene sulfonamide/formaldehyde resin. Although onycholysis has been attributed to reactions to toluene sulfonamide/formaldehyde resin, no published data firmly support this.[206,207] A new nail enamel compound has been introduced by Almay and Revlon. The toluenesulfonamide/folmaldehyde resin, the principal allergic sensitizer in nail enamels have been replaced by glyceryl tribenzoate. In addition, these new enamels are toluene-free. The new enamel also replaces dibutyl phthalate for glyceryl triacetate. This new plasticizer, polymer that prevents brittleness, provides longer wearability. Another very recent innovation is quick-drying suspensions. Two recent patents employing acetone and halogenated hydrocarbons provide for a reduction time from 50–70% over conventional nail enamel compositions without adversely affecting the other desirable properties of the coating. Environmental safe nail enamels are a real challenge for the cosmetic industry. Water-based nail polish with adhesion, gloss, and drying qualities will be developed.[208] A water-dilutable nail polish was developed containing a mixture of polyurethanes, vinyl, and acrylic ester.[209]

Yellow pigmentation of the nail plate, darkest at the distal end, occurs commonly in women who wear colored nail polish. Samman[105] reproduced this staining with the following colors: D&C Red No. 7, D&C Red No. 34, D&C Red No. 6, and FD&C Yellow No. 5 lake. Nail enamel removers are mixtures of solvents such as acetone, amyl, butyl, or ethyl acetate to which fatty materials may be added.[210] These can be irritating to the skin and can strip the nail plate. Cuticle removers contain alkaline chemicals, frequently sodium or potassium hydroxide to break the disulfide bonds of keratin. They should not be left on for prolonged periods or be used by people who are susceptible to paronychia. Cuticle removers are irritants.

"Sculptured nails" have become popular in recent years because they build an attractive artificial nail on the nail plate. Sculpture nails are prosthetic nails with a fresh acrylic mixture of methyl methacrylate monomer liquid and polymer powder. They are molded within a metabolized paperboard template on the natural nail surface to produce nails of desired thickness and length. When hardened, the template is removed, the prosthesis filed and the surface is polished. Acrylate sculptured nails are of two varieties: methacrylate monomers and polymers that are polymerized in the presence of hydroquinone in ordinary light and photo-bonded acrylate sculptured nails based on acrylates that are photobonded. Allergic reactions consist of paronychia, onychia, and severe and prolonged paresthesia. Fisher[211] reported that a patient developed a severe reaction to methyl methacrylate monomers resulting in permanent loss of all her fingernails. Fisher and Baran[212] reported that cyanoacrylates do not cross-react with other acrylates.

Unfortunately, irritant and allergic reactions to the liquid monomers as well as secondary infections may be painful and longlasting. Paronychia, onycholysis, onychia, and dermatitis of the finger and distant sites may occur. Fisher et al.[213] reported allergic sensitization to the methyl methacrylate monomers in sculptured nails. In 1974, the FDA banned the use of methyl methacrylate in these cosmetics. However, analysis of 31 products sold between 1975 and 1981 revealed that this monomer was present in nine of them.[214] Sensitization has also been reported to other monomers, and cross-reactions between acrylate monomers does occur.[215] Patch testing to 1.0–5.0% monomer in petrolatum or olive oil can help confirm the diagnosis of an allergic sensitization. Controls may be required if the patient responds to 5% and not to 1% of the monomer.

Performed plastic nails may be designed to cover the nail plate or extended tips. Their prolonged use causes mechanical damage to the nail, and those covering the entire nail plate may cause injury by occlusion.[216] Sensitization to *p*-tertiary butylphenol formaldehyde resin in the nail adhesive and tricresyl ethyl phthalate of the artificial nail has been reported.[217] Nail mending and wrapping kits often help women grow the longer nails they desire with few adverse reactions. A split nail can be repaired with cyanoacrylate glue with a negligible risk of sensitization. The repair is splinted with papers affixed by a nitrocellulose containing glue. These papers, or in some cases linen or silk, are wrapped over the free edge of the nail to protect it from trauma. Use of more sensitizing glues, of course, increases the risk of adverse reactions. The paper or cloth should not cover a large portion of the attached nail plate to avoid complications of occlusion.

68.11.11 Oral Hygiene Product

Dentifrices and mouthwashes are incriminated infrequently as causing allergic contact reactions. This may be due to the short exposure time these products have with the skin and mucous membrane under ordinary use situations. If sensitization occurs inside the mouth, patch testing on the skin usually shows a positive reaction. Many of the products contain detergents and are unsuitable for closed patch testing. To avoid irritant reactions, test open in the antecubital fossa and confirm positive results with tests in controls. To help

patients avoid further reactions, ingredient patch testing should be done. Fisher[218] reviewed concentrations for patch-testing ingredients found in toothpastes and mouthwashes. If the physician suspects allergic contact dermatitis and negative patch tests on the skin do not reflect mucosal sensitivity, ingredients may be incorporated in Orabase (Squibb). This material can be held against the inside of the lip for 24 h, and then examined for erythema. Reports of allergic sensitization to toothpastes primarily involve flavoring agents.[219] Cinnamicaldehyde has been the most frequent offender, because it was introduced in a relatively high concentration in toothpaste sold in several countries.[220] A case of contact dermatitis to cinnamic aldehyde resulted in depigmentation about the vermilion border.[33]

68.11.12 Personal Cleanliness Products

The action of bacteria on sterile apocrine secretions produces a characteristic odor. Labows et al.[221] reported lipophilic diptheroids as the organisms that produce unique axillary odors. Although deodorants are considered cosmetics, antiperspirants are regulated as OTC drugs as well as cosmetics. Many of these products have been reformulated in the last decade because of government regulations.[222] Hexachlorophene was banned because of its neurotoxicity and halogenated salicylanilides because of their photoallergic nature. Chlorofluorocarbon propellants were removed from aerosols because of their role in depleting the stratosphere of ozone. The chlorofluorocarbons have been replaced by hydrocarbon propellants-isobutane, butane, and propane, which are flammable. The FDA OTC Antiperspirant Review Panel recommended the removal of zirconium-containing chemicals from aerosol antiperspirants because of the potential for formation of granuloma in the lung. Sodium zirconium actate salts had caused granulomatous lesions in the skin of the axilla and have been removed antiperspirants.

Simple deodorants reduce the number of bacteria in the axilla. Most deodorants contain triclosan as an active ingredient. Triclosan is an antimicrobial agent used in soaps and shampoos. Although reports of skin damage and dermatitis due to triclosan exist, available statistics remain too limited to make a comprehensive judgment on the risk of triclosan use.[107,223] We recommend patch testing with 1–2% triclosan in petrolatum in suspected cases. Kampf and Kramer[223] note that when considering hand hygiene products a range of causative agents of irritant contact dermatitis exists spanning from 4% chlorhexidine gluconate (high) to alcohol-based hand rub formulations that include skin conditioners such as emollients (low). In fact, in an effort to reduce irritation and injury to the skin, the centers for disease control and prevention have supported the use of such alcohol-based formulations over more traditionally abrasive soaps and detergents.[223]

The OTC Review Panel published a list of aluminum and aluminum–zirconium chemicals permitted in antiperspirants. These chemicals are not regarded as sensitizers. Irritant reactions to aluminum salts in antiperspirants are common because of the environmental heat, moisture, and friction and the inflammation caused by shaving in the axilla. Dermal penetration of calcium salts and calcinosis cutis has been studied.[224] Allergic reactions are due to the other chemicals in the antiperspirants; most frequently the fragrance ingredients. Similarly, feminine hygiene sprays are primarily fragrance products that cause irritant reactions when sprayed at too close a range.

68.11.13 Baby Products

These products are marketed primarily to use on the skin and scalps of infants. Some experimental data suggest that infants are less easily sensitized than adults are. However, Epstein[225] reported that 44% of infants below 1 year of age could be sensitized to pentadecyl catechol, but that 87% of children above 3 years of age were sensitized in the same experiment. In clinical practice, allergic contact dermatitis is diagnosed infrequently in young children.[226] Patch testing with ingredients in standard concentrations may result in a higher incidence of irritant reactions in young children.[227] Because the diaper area is a frequent site of irritant contact dermatitis, careful attention should be paid to the products used in this area.

Generally, baby products are fragranced. Baby oil, talc, and cornstarch have simple compositions with little sensitizing potential beside the fragrances. Baby lotions or creams may contain fragrance, preservatives, lanolin, or propylene glycol, which are common sensitizers.[135] Propylene glycol, present in these lotions and the moistened towelettes marketed for cleansing the diaper area, is a common irritant. In the treatment of infants with diaper rash, it is important to examine the ingredients of the cosmetics used on the diaper area.

68.11.14 Bath Preparations

Adverse reactions to bubble bath reported to the FDA include skin eruptions, irritation of the genitourinary tract, eye irritation, and respiratory disorders.[228] The genitourinary tract reactions in children have been the most serious; many children have been subjected to extensive urologic workups before the cause was established. The skin eruptions are assumed usually to be irritant reactions due to the detergent content of this cosmetic.

68.11.15 Other Skin Care Preparations

68.11.15.1 Depilatories

Most depilatories today contain mercaptans such as calcium thioglycolate 2.5–4.0% in conjunction with an alkali to bring the pH to between 10 and 12.5.[210] The keratin of the cortex is more vulnerable before it emerges from the follicle, and depilatories attack it there leaving a soft rather than sharp end. For this reason, the use of depilatories in place of shaving can prevent pseudofolliculitis barbae in some black men. Powdered facial depilatories, produced for beard removal, contain barium or strontium sulfide because these chemicals

are quicker acting. Unfortunately, these chemicals cause more irritation and produce an unpleasant odor. To use the less malodorous thioglycolate depilatories for coarser beard removal, hair accelerators such as thiourea, melamine, or sodium metasilicate are added. Depilatories cannot be patch tested directly and these thioglycolates are seldom sensitizers.

68.11.15.2 Epilating Waxes

Epilating waxes are usually warmed to soften, and they harden and enmesh the hair after application. When the wax is pulled off, the hair is removed by the root. Some modified waxes do not have to be warmed and can be applied with a backing material. These cosmetics may contain beeswax, rosin (colophony), fragrance, or rarely benzocaine as potential sensitizers.[210] The problems usually seen with these epilating cosmetics are due to mechanical irritation.

68.12 COSMETIC INTOLERANCE SYNDROME

Fisher[229] coined the term "status cosmeticus" for the condition in which a patient is no longer able to tolerate the use of many or any cosmetic on the face. Patients may complain of itching, stinging, facial burning, or discomfort. This group seriously challenges our diagnostic skills as well as our ability to be empathetic because the severity of patients' symptoms does not match with objective signs of disease. Most of these patients have only subjective symptoms, but some may have mild inflammation. The cosmetic intolerance syndrome is not a single entity, but rather a symptom complex due to multiple factors, exogenous and endogenous.[35] Therefore, these patients need a thorough history, physical examination, and workup. Some patients have occult allergic contact dermatitis, allergic photocontact dermatitis, or contact urticarial reactions, and the causal agents are documented by careful clinical review and patch testing.

Others who have a seborrheic or rosacea diathesis with or without inflammation seem to have flared this condition by overusing cleansing creams and emollients. Both of these conditions may be accompanied by facial erythema or scaling. Some patients require anti-inflammatory therapy, as do atopic patients who develop this state. Fisher recommended that, whenever possible, irritating chemicals should be avoided by these patients.

When the offending agent cannot be found, prolonged elimination of cosmetics seems to help some women who after 6–12 months or more are able to gradually return to the use of other cosmetics (Table 68.8). Addition of skin care products should be made one at a time and no more frequently than every 2 weeks. The final program should be simple and limited in the number and frequency of cosmetics used. Goldenberg and Safrin[230] suggested that stinging effects of cosmetics irritants may be neutralized by anti-irritants. They proposed three possible mechanisms of action of anti-irritants: to complex the anti-irritant, to block the reactive sites in the skin, and to prevent physical contact with the skin.

TABLE 68.8
Management of Patients Who Are Intolerant to Cosmetic Usage

1. Examine every cosmetic and skin care agent
2. Patch and photopatch test to rule out occult allergic and photoallergic contact dermatitis, or contact urticaria
3. Limit skin care to
   Water washing without soap or detergent
   Lip cosmetics
   Eye cosmetics (if the eyelids are not symptomatic)
   Face powder
   Glycerol and rose water as moisturizer (only if needed)
   6–12 months of avoidance of other skin care agents and cosmetics
4. Watch for and test, if necessary, depression and other neuropsychiatric aspects

68.13 OCCUPATIONAL DERMATITIS: HAIRDRESSERS

Cosmeticians may perform a variety of personal care tasks including hair grooming, manicuring, and applying makeup. It is primarily the hair care tasks that account for the high rate of occupational hand dermatitis. Cronin[231] noted that beauticians frequently develop a dry, scaling dermatitis over the metacarpophalangeal joints, however, individuals vary in their ability to react to irritants.[232] Novice beauticians and those in training are required to shampoo many customers each day, and the resulting irritation is frequently the initial cause of hand dermatitis. These hairdressers have a good chance of improving as they learn to protect and lubricate their hands. However, patients with atopic eczema may have a particularly difficult time with hand dermatitis as hairdressers, although we do believe they should not be barred from this career. Young atopic patients who are contemplating career choices should be apprised of the occupational hazards of hairdressing.

When beauticians with hand dermatitis are patch tested at different centers, the percentage of reactions varies. PPD is usually the leading offender when allergy is present.[233] Frosch and coworkers[143] reported that the major contact sensitizer of hairdressers in Europe was GMT. Sensitization is at least as frequent to PPD; in some countries (Germany, United Kingdom, Spain) sensitization frequencies were high. This has to be emphasized in comparison to the relatively low frequencies to AMT. The recently introduced acid permanent waves pose a higher risk of sensitization to hairdressers than the alkaline permanent waves that have been used since the early 1940s. The low figures for GMT sensitization in some centers may be explained by lower usage in salons or by more careful handling. In Denmark, most hairdressers wear gloves when dyeing and permanent waving. In Germany, most hairdressers protect their hands only against hair dyes. There is still a strong prejudice against the use of gloves in this occupation. Guerra et al.[234] confirmed this attitude in Italy: only 12.5% of 240 hairdressers wore gloves for permanent waving, whereas 51% wore them for hair dying. This group

found a relatively low sensitization to GMT, attributing this to its infrequent usage in Italy. They demonstrated that vinyl gloves may not always suppress the reaction to GMT in sensitized individuals. They found three of eight patients patch tested with GMT through vinyl gloves were positive after 3 days. Better plastic materials must be looked for, if GMT continues to be used in European salons. Furthermore, it must be kept in mind that wearing gloves over a prolonged time poses its own risks. The primary goal in the prevention of occupational dermatitis must be reduction of exposure to highly sensitized agents. They reported that GMT was the number one sensitizer. After the series described by Storrs,[142] the German CDRG reported sensitization to GMT in 38% of 87 patients in 1989, and in 31% of a second series of 178 patients.[235] Uter et al.[236] found that between 1992 and 1998 where products that contained GMT were removed from the market in Germany, the fraction of hairdressers with positive reactions to GMT decreased from 45 to 20%.

Hairdressers need to be instructed to handle this type of permanent wave with caution. Direct skin contact should be avoided. Gloves and improved handling technique may lead to a decrease in the frequency of sensitization, which may, in comparison to hair dyes, be acceptable to this occupational group.

Rietschel et al.[237] and other investigators have shown that allergens can penetrate gloves. Fisher reported that polyethylene laminate glove (4-H glove; Safety 4 Company, Denmark) protect allergic patients from epoxy resin and acrylic monomers. McClain and Storrs[238] in a placebo-controlled double-blind patch test study reported that 4-H glove was effective in preventing allergic contact dermatitis in GMT-sensitized volunteers, protecting 4 of 4 patients after an 8 h exposure and 2 of 3 after 48 h. Melstran et al.[239] provide extensive documentation about protective gloves. Frosch and coworkers[143] found PPD, the second sensitizer very close to GMT. However, PPD derivatives were considerably lower and ranged from 375. In the Italian study, the figures were similar for PPD but higher for the derivatives. Frosch and colleagues were unable to conclude that ONPPD had the lowest sensitization risk. They stated that pyrogallol and resorcinol are the least frequent sensitizers in the hairdressers' series.

Nickel, preservatives such as (cloro) methylisothiazolinone and formaldehyde, surface active agents such as cocamidopropylbetaine, and hydrolyzed animal proteins, as well as perfume ingredients, may also be responsible for dermatitis in hairdressers. To work-up hairdressers with hand eczema, we use the standard hairdressers screening series. This series includes resorcinol, *p*-toluenediamine sulfate, glyceryl monothioglycolate, ammonium thioglycolate, ammonium persulfate, *p*-aminodiphenylamine hydrochloride pyrogallol, and *o*-nitro-*p*-phenylenediamine. At the same session, we apply 1.0% glyceryl thioglycolate in petrolatum, and pieces of the hairdresser's protective glove applied on both sides.

Hairdressers who are nickel-sensitive also have a serious challenge. There is evidence that permanent solutions may leach nickel out of metal objects.[240] Fastidious care in the selection of stainless steel tools and use of dimethyl glyoxide for testing pins, clips, and other paraphernalia allows some patients to continue in this career. Hairdressers with allergic contact dermatitis need to be told that protective gloves may not provide an absolute barrier to allergens.[143]

Tomb and coworkers[241] reported a young hairdresser who developed acute periorbital eczema and marked edema of eyelids, lip erosions, and eczema of her fingertips to instant glues used to attach false hair. Patch test was strongly positive to ethyl cyanoacrylate adhesive.

Ingredient labeling of retailed cosmetics in the United States has greatly aided dermatologists in caring for patients with contact dermatitis. There is no regulation requiring similar labeling for cosmetics used in beauty salons. However, preventative strategies for occupational skin diseases in hairdressers are known and can be rarely successful.[242]

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69 Decreasing Allergic Contact Dermatitis Frequency through Dermatotoxicologic-and Epidemiologic-Based Intervention?

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Despite implementation of regulations to reduce exposure to sensitizers, allergic contact dermatitis (ACD) remains a burden worldwide. Sensitizers leading to ACD include metals, plants, cosmetics, rubber compounds, and medicines.[1,2] In the workplace, contact dermatitis (irritant and allergic) accounts for 40% of all occupational illnesses (excluding injury) and one-fourth of time lost from work.[3] Thus, the economic burden of contact dermatitis secondary to occupational exposures is extensive due to work hours lost and doctor visits. Several countries have implemented legislations in an attempt to reduce the exposure of chemicals leading to ACD and studies examining trends in patch test reactivity have been conducted to determine whether these legislations have been effective.

Nickel, a metal found frequently in jewelry, is a common cause of ACD in industrialized countries.[4] Jensen et al. evaluated whether a Danish regulation implemented in 1992 that decreased nickel exposure had an impact on the prevalence of nickel sensitization in girls with pierced ears.[5] They provided questionnaires and patch testing to 534 girls from seven high schools and two production schools (mean age 18.8 years, range 17–22) and 427 fifth and sixth grade girls from 12 public schools (mean age 12.4 years, range 10–14), to examine girls who would have had their ears pierced before and after implementation of the nickel exposure regulation, respectively. Both the older-aged girls (with and without ear piercings) and girls who had their ears pierced before 1992 had an increased prevalence of nickel sensitization. Specifically, 17.1% of the older girls demonstrated a positive patch test to nickel compared to 3.9% of the younger girls. Also, the prevalence of nickel sensitization was significantly higher in girls with their ears pierced before 1992 compared with girls with unpierced ears (OR 3.3, $p = .004$). Thus, the authors conclude that the study has demonstrated a decrease in nickel sensitivity in Denmark after implementation of the nickel regulation and that currently, ear piercing does not seem to be a critical factor for the development of nickel contact allergy.

Although the investigators demonstrated a decreased prevalence of nickel sensitization in younger girls, note that only 71.4% of the young girls and 51.5% of the older girls recruited for the study were actually patch tested; thus, there may be a selection bias in those participating. Additionally, there may be a memory or reporting bias in answering the questionnaire. In girls who reported prior positive patch test results to nickel, the authors did not document whether the patient's report of the patch tests results were confirmed. Interestingly, the authors found only a small difference in mean nickel exposure time, a factor that may be critical in sensitization to nickel, between individuals testing positive and negative and between the older and younger girls; however, they did not explain how nickel exposure time was determined and did not show their data with regard to these results. Additionally, even though they attempted to account for influences of socioeconomic status on the data by including girls from high schools and production schools, note that socioeconomic status may be a confounding factor in that girls from a higher socioeconomic status may more easily be able to purchase jewelry that does not contain nickel. Overall, evidence supports a decreased prevalence of ACD secondary to nickel as no other explanation can be proven.

Like nickel, fragrances and hair products also account for a large portion of sensitization leading to ACD. A high incidence of sensitization in hairdressers to an ingredient of acid perms, glyceryl monothioglycolate (GMT), led to withdrawal of products containing GMT from the German market in 1992. Uter et al. analyzed patch test data from 1336 hairdressers from 1992 to 1998 to confirm the obvious—whether the withdrawal of GMT was effective in reducing ACD.[6] The proportion of hairdressers testing positive to GMT fell from

45% in 1992 to less than 20% in 1997 and 1998; thus, providing evidence that withdrawal of GMT was effective. The authors account for the "less than 20% in 1997 and 1998" by the fact that hairdressers may still be exposed from small manufacturers and importers who have not removed GMT from their products. They also note that some improvement may be secondary to increased skin protection at work in recent years (by use of gloves, etc.), but this is less likely because the proportion of other typical allergens found in hairdressers did not change. Note, however, that there may be a selection bias in the hairdressers who sought patch testing during this study period and there may also be a reporting bias in the clinics that chose to report the results of the patch testing to the Information Network of Departments of Dermatology (the institution that the data was gathered from). Interestingly, ammonium thioglycolate, a perming agent whose relative use decreased with the introduction of GMT but which has been repopularized to replace GMT, has caused even fewer reactions than its prior introduction. This raises the question of whether other factors are involved in eliciting ACD. The ammonium thioglycolate ACD story resists clinical clarification because of its patch test irritancy and lack of provocative use test data that would permit ascertainment of whether the positives represent irritation or allergy.

Buckley et al. examined the pattern of fragrance allergy in 25,545 (15,005 female and 10,450 male) patients from 1980–1996 in London, England.[7] Positive patch test reactions to "fragrance mix" remained relatively constant throughout the period, ranging from 6.1 to 10.9% (mean 8.5) in women and from 5.1 to 12.9% (mean 6.7) in men; however, the patch test reactions to individual fragrances varied. Specifically, positive patch test reactions to balsam of Peru decreased at 5% per year ($p < .001$, 95% CI 2.4–6.9), hydroxy-citronellal decreased 5% yearly ($p = .032$, 95% CI 0.5–10.2), cinnamic aldehyde decreased by 18% yearly ($p < .001$, 95% CI 14.3–21.0), and cinnamic alcohol decreased by 9% yearly ($p < .001$, 95% CI 5.2–12.9); while oak moss increased 5% yearly ($p = .001$, 95% CI 2.2–8.7), isoeugenol increased 5% yearly ($p = .005$, 95% CI 1.5–9.2), alpha-amyl cinnamic aldehyde increased 10% yearly ($p = .042$, 95% CI 0.3–20.5), and eugenol and geraniol remained relatively constant. While all of these values are statistically significant according to a p-value $< .05$, the data for alpha-amyl cinnamic aldehyde and hydroxyl-citronellal may not be significant because 95% CI for these data include 1. Also note that the overall decline in positive reactions to cinnamic alcohol was not gradual, instead there were alternating spikes and troughs with each year of study. Overall, the investigators feel that reductions in concentrations of certain fragrances in cosmetics and other products has resulted in decreases in population sensitivity to those ingredients; however, increases in the quantity of other perfume ingredients has left the overall frequency of fragrance allergy unchanged. This is confusing because the authors do not document which of the individual fragrances has been reduced in products to make the correlation.

Chromate is another human allergen. Avnstorp investigated dermatitis due to chromate found in cement in 1840 workers exposed before and after implementation of industrial chromate reduction with ferrous sulfate in Denmark.[8] The prevalence (8.9% in 1981–1.3% in 1987) and the approved number of claims of allergic cement eczema decreased after implementation of chromate reduction; however, the status of those already affected by ACD secondary to chromate did not change. Evidence of concomitant sensitivities to cobalt and rubber chemicals may in part explain the unfavorable prognosis of those already affected. Also, individual preventative measures, such as the use of gloves and barrier creams, were not found to influence development of ACD. Although the mechanism behind developing ACD is thought to be related to exposure to sensitizers, interestingly, Avnstorp found that neither a change of occupation nor retirement changed the medical prognosis of ACD in comparison with remaining in the original occupation. Thus, it is believed that some workers who stay with the same occupation may develop immunologic tolerance or hyporeactivity induced by repeated skin irritation. Although this study brings up interesting points in addition to finding that chromate reduction has decreased the prevalence of ACD, note that the workers removed the patches after 48 h themselves, allowing for some limitations in the data. Also, as in the other studies, there may have been a selection bias of the participants chosen to be in the study, and confounding factors may include age of the participants, varying exposures to cement (chromate), and duration of exposure.

TABLE 69.1
Changes in Allergic Contact Dermatitis Frequency before and after Implementation of Regulations

Sensitizer	Baseline	"Current"[a]	Comment	Reference
Nickel	17.1%	3.9%	Prevalence before and after 1992, OR 3.3 ($p = .004$)	Jensen et al.
GMT	45%	20%	—	Uter et al.
Fragrance	—	—	Overall, fragrance allergy unchanged (see text for details)	Buckley et al.
Chromate	8.9%	1.3%	—	Avnstorp
Thiuram	—	—	Increase from 1983–1988 to 1989–1995 (OR 2.55, $P = .01$). Decrease after 1995, but not statistically significant%	Gibbon et al.

[a] "Current" refers to most recent time frame accounted for in the respective study. See text for details.

As an example of increasing and decreasing frequency in ACD, Gibbon et al. examined patch test results to thiuram, a rubber additive found in latex gloves, in 450 healthcare workers (mean age 33 years, range 17–65) and 630 housewives (mean age 40 years, range 18–79) in the U.K. over a 16-year period (1983–1998).[9] There was an increase in the incidence of thiuram-positive patch tests from 1989 to 1995 compared to 1983–1988 in healthcare workers (OR 2.55, $p = .01$, 95% CI 1.25–5.20), in contrast to housewives who demonstrated no significant change during the same period. However, in the healthcare workers group, there was a decreasing trend in thiuram allergy after 1995, but the change was not statistically significant. The authors believe that the decrease in the incidence of thiuram allergy in the latter half of the 1990s may indirectly reflect the National Health Service's legislation to improve the quality of gloves in an attempt to decrease latex protein allergy. The exact legislation and the date that it was implemented were not documented. Like the other studies, there may have been a selection bias, because the patients patch tested came from a hospital that receives numerous tertiary referrals. Another issue that must be raised is whether housewives were an appropriate control for the healthcare workers since their exposure to thiuram or natural latex gloves is unknown and possible significantly less.

Overall, the evidence suggests a decreasing trend of ACD with appropriate formulation changes following toxicologic and epidemiologic information. However, exposure to the chemical via small manufacturers or imported products that do not follow regulation may still be possible. Thus, stricter control (legislation?) must be implemented to decrease sensitizers from products worldwide. In the mean time, exact ingredient labeling and a decrease in the number and level of potential sensitizers in products should help. Additionally, further investigation of the mechanism of ACD may give new insight as to its prevention and treatment. Until enlightened toxicologic and epidemiologic evidence based judgment (and appropriate regulation) are implemented, ACD secondary to nickel, hair products, fragrances, chromate, and rubber additives, among other chemicals, will continue to affect masses of people.

In any instance, rational judgments will be improved when more population-based data are generated as a back up for the case and patch test frequency studies. Nielsen et al. provide an excellent model of the former in their study of the incidence of allergic contact sensitization in Danish adults between 1990 and 1998 and Menne et al. review the previous population based ACD literature.[10,11] Additionally, Menne and Whalberg comment on the failure of ACD risk assessment and stress the need for epidemiologic monitoring to correct these failures.[12]

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70 Cutaneous Corticosteroid-Induced Glaucoma

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70.1 GLAUCOMA

Glaucoma is a leading cause of irreversible blindness in the world. World Health Organization published in 1995 that glaucoma was responsible for the blindness in 5.1 million persons or 13.5% of all blindness in the world.[1]

Glaucoma, an optic nerve neuropathy, is a disease that leads to optic atrophy and progressive loss of the visual field. The optic atrophy and visual field loss are secondary to ganglionar cell death and these are developed when the pressure is higher than that tolerated by each eye.[2]

70.2 CORTICOSTEROID AND GLAUCOMA

Glaucoma can be classified based on its etiology or mechanisms of outflow obstruction. Glaucoma in a patient using corticosteroid can be classified as a corticosteroid-induced glaucoma if no other systemic condition that causes glaucoma is present or after an initial test with corticosteroid glaucoma develops in the contralateral eye, or if after the corticosteroid is discontinued the pressure does decrease. Glaucoma is associated with a trabecular obstruction, so in corticosteroid users it is associated with a reduction in facility of aqueous outflow.[3,4]

Different sources of corticosteroids are responsible for the rise in intraocular pressure (IOP).[5] The first division must be between the exogenous and the endogenous. Exogenous include: ocular (eyedrops, ocular ointments, and inadvertent administration to the eye from the lids or face), periocular injection, and systemic (oral, injection, topical (skin), aerosol (inhalation)/spray nasal).[6] Endogenous include: adrenal hyperplasia, adrenal adenoma or carcinoma, and ectopic adrenocorticotropic hormone (ACTH) syndrome[5,7–9] (Table 70.1).

The proposed mechanism in corticosteroid-induced glaucoma includes morphological and functional changes in the trabecular meshwork system and is similar to the pathogenesis of primary open-angle glaucoma. An important point is the differentiation between glaucoma and ocular hypertension; glaucoma is characterized by not only rise in IOP (hypertension) but also excavation and degeneration of the optic disk and damage to nerve fiber bundles.[10]

Approximately 18–36% of the general population show a 5 mm Hg or more increase in IOP after ophthalmic administration of topical corticosteroids.[11]

70.3 CUTANEOUS CORTICOSTEROID-INDUCED GLAUCOMA

Corticosteroids play an important role in the treatment of inflammatory or immune-mediated skin diseases. Dermatologists traditionally avoid even short-term cutaneous corticosteroid (except hydrocortisone) in the treatment of eyelid dermatitis, even though some patients with chronic eyelid dermatitis resolve after short-term higher potency cutaneous corticoids (personal observation). These patients usually present with itching and red eyelids (e.g., atopic dermatitis, contact dermatitis, contact urticaria, rosacea, seborrhea, and psoriasis).[12]

Topical application of corticosteroids to the skin may cause open-angle glaucoma, for example, after long-term corticosteroids ointment application to the periorbital region for treatment of atopic dermatitis or vitiligo vulgaris.[13] Even cosmetic products such as facial lotions and creams with corticosteroid components, may cause ocular hypertension after prolonged application to the periocular region.[8,14]

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The ability of certain topically applied corticosteroids to raise IOP in susceptible individuals, particularly in open-angle glaucoma patients, require care in the ophthalmologic and dermatologic practice.[15] Others factors associated with glaucoma are family history, ocular hypertension, migraine, myopia, race (African-American population), untreated systemic hypertension, diabetes mellitus, and corticosteroid responsiveness.[16–22]

TABLE 70.1
Classification of Corticosteroids—Different Administrations Causing Glaucoma[5–9]

Administration Types	
Exogenous	**Endogenous**
Ocular/Periocular	Adrenal hyperplasia
Ointment	Adrenal adenoma/carcinoma
Cream	Ectopic ACTH syndrome
Eyedrops	
Subconjunctival (injection)	
Retrobulbar (injection)	
Periocular injection	
Topical on the Skin	
Ointment	
Cream	
Injection	
Nasal	
Spray	
Aerosol	
Oral/injection	

70.4 CUTANEOUS CORTICOSTEROID INDUCED—CASES IN LITERATURE

Sixteen cases of facial, including periocular (eyelids), exposure to topical corticosteroids leading to increased IOP and glaucoma are outlined in the Tables 70.2 and 70.3.[8,13,14,23–28]

Sixteen patients provide only limited opportunity to define a clinical entity. Note that most patients utilized the corticoid for long periods (2–15 years), and at least once daily (between one and three times daily). The shortest exposure was 2 years (described by Katsushima, 1995).[13] In most patients IOP either decreased spontaneously or with medical treatment after the corticoid was suspended (Table 70.3). Eight patients had permanent optic disk damage.[5–9,14]

70.5 CUTANEOUS CORTICOSTEROID INDUCED—SUMMARY

IOP changes associated with the use of cutaneous corticoids appear uncommon, but an under-reporting artifact is likely. The cases highlighted earlier involved chronic rather than acute corticoid exposure.

Many hypotheses attempt to explain how cutaneous corticoids increase IOP: penetration through the eyelid due to the thin facial skin, improper application technique, self-inoculation into the eye, and perspiration leading to seeping of corticosteroid in the eye. Possible source of corticoids affecting IOP are described in Table 70.1.[24]

A rise in IOP may be noted as early as 1 week after initiating ophthalmic glucocorticoid treatment, or it may

TABLE 70.2
Dermatologic Description of Patients Using Corticoid Ointment/Cream on the Face

Authors	Eye	Eye Symptom	IOP[a]OD	IOP[a]OS	VA-OD[a]	VA-OS[a]	Cause LVA[a]	Treatment OD[a]	Treatment OS[a]
Cubey[14]	2	Haloes OS	60	60	20/20	20/20	—	M;[a] S*	M;[a] S[a]
Zugerman[8]	1	Haloes OS	NI	55	—	20/100	Corneal edema	M[a]	—
Nielsen[24]	2	Routine	30	32	20/20	20/20	—	M[a]	M;[a] S[a]
Nielsen[24]	2	Blurred vision	46	32	20/200	20/40	Damage OD[a]	M[a]	M[a]
Vie[25]	2	Blurred vision	43	63	NI	Amaurotic	Damage OD[a]	M[a]	NI
Eisenlohr[26]	2	Blurred vision and haloes	55	57	20/20	20/25	—	M;[a] S[a]	M;[a] S[a]
Aggarwal[23]	2	Blurred vision OS	44	74	20/15	20/200	Damage OD[a]	M[a]	M;[a] S[a]
Aggarwal[23]	2	Blurred vision	28	54	20/15	20/120	Damage OD[a]/cataract	M[a]	M;[a] S[a]
Aggarwal[23]	2	Blurred vision	32	27	20/80	20/60	Damage OD[a]/cataract	M[a]	M[a]
Aggarwal[23]	2	Routine	28	28	20/15	20/15	—	Suspension C.[a]	Suspension C.[a]
Aggarwal[23]	2	Routine	26	26	20/20	20/20	—	Suspension C.[a]	Suspension C.[a]
Katsushima[13]	1	NIA	NIA	35	NIA	NIA	—	NIA	NIA
Katsushima[13]	1	NIA	NI	50	NIA	NIA	Damage OD[a]	NIA	S[a]
Katsushima[13]	1	NIA	68	NIA	NIA	NIA	Damage OD[a]	S[a]	NIA
McLean[27]	2	Blurred vision/haloes	40	40	20/30	20/40	Damage OD[a]/cataract	M;[a] Facectomy	M;[a] Facectomy
Schwartzenberg[28]	2	Blurred vision	40	36	20/30	20/30	—	M[a]	M;[a] S[a]

Note: NIA, not provided in the abstract; NI, not provided in the article.

[a] Eye, refers to the number of eyes involved in each patient; IOP, intra ocular pressure; VA, visual acuity; LVA, low visual acuity; OD, right eye; OS, left eye; Cause LVA, show the reason why some patients have vision below 20/40; M, medical therapy; S, trabeculectomy; Damage, damage of the optic disk based on the description of the article.

TABLE 70.3
Ophthalmologic Description of Patients Using Corticosteroids Ointments/Cream on the Face with Raised Intra-Ocular Pressure

Authors	Year	Age	Gender	Derm. Disease	Corticoid Type	Cort. Conc.[a] (%)	Duration (Years)	Local[a]
Cubey[14]	1976	22	Male	Facial eczema	Fluocinolone acetonide oint	0.01	7	Face + eyelid
Zugerman[8]	1976	30	Male	Contact dermatitis eyelids	Triancinolone cr.	0.50	8	NI
Nielsen[24]	1978	68	Female	Periorbital eczema	Prednisone ointment	0.50	2	Eyelid
Nielsen[24]	1978	80	Female	Periorbital dermatosis	Triamcinolone acetonide	0.10	3	Face + eyelid
Vie[25]	1980	29	Female	Atopic dermatitis	Fluorcortison cream	0.5	15	Face + eyelid
Eisenlohr[26]	1983	33	Female	Cosmetic irritation	Betamethasone cream	0.10	4	Ni
Aggarwal[23]	1993	24	Male	Facial eczema	Desoxymethasone cream	0.25	2	Face + eyelid
Aggarwal[23]	1993	23	Male	Periorbital atopic eczema	Hydrocortisone cream	1	12	Periorbital
Aggarwal[23]	1993	25	Male	Periorbital atopic eczema	Hydrocortisone cream and others	1	4	Periorbital
Aggarwal[23]	1993	13	Male	Atopic dermatitis	Clobetasone butyrate cream	0.05	12	Face+trunk
Aggarwal[23]	1993	21	Female	Facial eczema	Betamethasone cream	0.10	10	NI
Katsushima[13]	1995	NIA*	NIA	Vitiligo vulgaris	Fluocinolone acetonide ointment	0.025	2	Periorbital
Katsushima[13]	1995	NIA*	NIA	Atopic dermatitis	Methylpredinisolone ointment	0.5	14	Periorbital
Katsushima[13]	1995	NIA*	NIA	Atopic dermatitis	Betamethasone valerate ointment	0.12	2	Periorbital
McLean[27]	1995	30	Male	Discoid eczema	Hydrocortisone cream	2.50	4	Face
Schwartzenberg[28]	1999	22	Female	Eyelid dermatitis	Diflucortolone valerate cream	0.10	10	Eyelid

Note: NIA, not informed in the abstract; NI, not informed in the article.

[a] Corticoid type was related to the corticoid used for the longest period; duration was reported considering all drugs used by the patient.

occur after months to years. The latent period before the IOP begins to rise and the magnitude of the pressure elevation are related to drug potency, administered dose, frequency of administration, route of administration, presence of other ocular and systemic disease, and most important, the patient's responsiveness.[5]

This chapter highlights 28 eyes, with a rise in IOP during the treatment of a facial disease; of these 28 eyes, 10 required surgery for glaucoma, despite discontinuing corticoid therapy. Hydrocortisone appeared three times. In one case prolonged use led to glaucoma and ultimately required trabeculectomy.[23] This patient used only hydrocortisone as monotherapy, and the other two were treated with hydrocortisone and other topical corticoids (Tables 70.2 and 70.3). Only one (diabetic) patient had an identified risk factor for glaucoma noted before the onset of increased IOP or other eye diseases[24] (Table 70.3).

Prolonged use of corticoids produces a type of glaucoma similar to chronic simple glaucoma (open-angle glaucoma).[29] Otherwise, ocular hypertension and glaucomatous field defects are more likely to develop in corticosteroid-responsive individuals than in nonresponsive individuals.[10] This may explain why in some patients corticoid discontinuation did not lead to decreased pressure. There is evidence of a genetic predisposition to corticoid-induced glaucoma, although the mode of inheritance is unknown.[30–32]

Although, it should be understood that all these patients had glaucoma associated with corticosteroid use, it does not mean that all of them would not have glaucoma if they did not use corticosteroids.

Patients should be seen by an ophthalmologist prior to long-term use of corticosteroids, and if they present any ocular complaint especially haloes and blurred vision which suggest increased IOP.

In managing patients with refractory eyelid or periocular inflammatory disease, we empirically utilize a prophylactic regimen: ophthalmologic screening for IOP before cutaneous dosing, approximately 2 weeks later, and every 2–3 months. This would appear to provide a large safety margin. The shortest period of exposure in this dermatology literature was 2 years, although ophthalmic literature demonstrates IOP increases as soon as 2 weeks.[5] Our experience has been

TABLE 70.4
Guideline to Preventing Glaucoma from Cutaneous Corticoid Dosing

1. Baseline: ophthalmic examination (including IOP).
2. Repeat IOP approximately 2 weeks after initiating corticoid.
3. Repeat IOP measurement approximately every 2–3 months.
4. Taper corticoid to the lowest potency practical as soon as clinical response permits.
5. Ocular symptoms (redness or blurred vision) mandate ophthalmic examination.

that most chronic dermatitis and psoriasis respond to mild or mid (and rarely higher) potency corticoids, and that the drug can be discontinued or decreased in potency in weeks (Table 70.4).

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71 Evaluating Efficacy of Barrier Creams: *In Vitro* and *In Vivo* Models

Hongbo Zhai and Howard I. Maibach

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71.1 INTRODUCTION

Skin-protection products are used in the occupational field to protect the skin against hazards from the workplace. One major measure utilizes the barrier creams (BC) in the prevention of contact dermatitis (CD) including both of irritant contact dermatitis (ICD) and allergic contact dermatitis (ACD), which is a major occupational disease, with a significant medical, economical, and social impact. However, BC are recommended only for low-grade irritants (water, detergents, organic solvents, cutting oils) (Frosch et al., 1993a; Zhai and Maibach, 1996b; Wigger-Alberti and Elsner, 1998) and cannot replace other protective measures such as gloves. Their efficacy in reducing the developing ICD and ACD have been documented in *in vitro* and *in vivo* experimental studies (Frosch et al., 1993a; Lachapelle, 1996; Zhai and Maibach, 1996a, 1999, 2002; Wigger-Alberti and Elsner, 1998, 2000a,b; Maibach and Zhai, 2000). However, inappropriate BC application may induce a deleterious rather than a beneficial effect (Goh, 1991a,b; Frosch et al., 1993a,b,c,d; Treffel et al., 1994; Zhai and Maibach, 1996b; Lachapelle, 1996). Two major reasons might generate these divergent results: one is the design defect of the BC; another possibility relates to the testing models. Thus, the methodology is important and hence the accuracy of results depends on the choice of proper models.

In vitro and *in vivo* methods have been developed to evaluate efficacy, and this subject has been reviewed (Frosch et al., 1993a; Lachapelle, 1996; Zhai and Maibach, 1996a, 1999, 2000, 2001, 2002, 2004; Wigger-Alberti and Elsner, 1998, 2000a,b; Maibach and Zhai, 2000). This updated chapter from Zhai and Maibach (2004) briefly introduces recent methodologies and related data.

71.2 TESTING METHODOLOGY

71.2.1 *In Vitro* Methods

Treffel et al. (1994) measured the effectiveness of BC on human skin against dyes (eosin, methylviolet, and oil red O) with varying n-octanol/water partition coefficients (0.19, 29.8, and 165, respectively). BC effects were assayed by measurement of the dyes in the epidermis of protected skin samples after 30 min. Some BC showed efficacy but several revealed data contrary to manufacturers' claims. No correlation existed between the galenic (pharmaceutic) parameters of the assayed products and protection level, indicating that neither the water content nor the consistency of the formulations influenced effectiveness.

Fullerton and Menne (1995) tested the protective effect of ethylenediaminetetraacetate barrier gels against nickel contact allergy. Thirty milligrams of barrier gel was applied on the epidermal side of the skin *in vitro* and a nickel disk applied above the gel. After 24 h, the nickel disk was removed and the epidermis separated from the dermis. Nickel content in epidermis and dermis was quantified by adsorption differential pulse voltammetry. The amount of nickel in the epidermal skin layer on barrier gels treated skin was significantly reduced compared to the untreated control.

Shah and Kirchner (1997) evaluated moisture penetration through a thin film of skin protectants *in vivo* and *in vitro* utilizing Fourier transform infrared spectroscopy. Petrolatum offered some protection against water penetration; a hydroactive polymer system (protectant) prevented moisture penetration.

Goffin et al. (1998) assessed the efficacy of BC to surfactants and organic solvents with shielded variants of corneosurfametry and corneoxenometry method. Petrolatum exhibited the best protection being a blocker for sodium

Modified from *Dermatotoxicology*, 6th Edition.

lauryl sulfate (SLS) and also provided protection against hexane-methanol.

Zhai et al. (1999a) utilized an *in vitro* diffusion system to measure the protective effect of three quaternium-18 bentonite (Q18B) gels to prevent 1% 35S-SLS penetration into human cadaver skin. The accumulated amount of 35S-SLS in receptor cell fluid was counted to evaluate the efficacy of the model Q-18B gels over 24 h. These test gels significantly decreased SLS absorption when compared to the unprotected skin control samples. Protection effect (percent) of three Q18B was 88%, 81%, and 65%, respectively.

van Der Bijl et al. (2000) conducted an *in vitro* study of the permeability of tritiated water through fresh and frozen human skin in the presence and absence of two different BC in a flow-through diffusion system. Buffer/tritiated water was collected from the acceptor chambers at 2-h intervals for a total of 20 h and counted in a liquid scintillation counter. Both BC lowered the average flux rates of tritiated water through fresh and frozen skin, but no significant differences could be detected between the two.

zur Muhlen et al. (2004) carried out a 3D skin model tests following an *in vivo* test (Repetitive Occlusive Irritation Test) (ROIT) to analyze the mode of action of the protection cream. This model could provide insights on the protective mechanism of the tested BC. It also showed that the multiple emulsion reduces cell damage after treatment with sodium dodecyl sulfate (SDS).

Barbadillo et al. (2006) introduced a simplified method to evaluate the efficacy of BC in an efficient manner. Human cadaver skin was fastened onto liquid scintillation vials as a static model. The larger surface area (~1.75 cm^2) was ideal to measure the skin's transepidermal water loss and capacitance utilizing appropriate instruments. Five protective agents/formulations were applied to skin prior to lipid extraction. Results showed that only petrolatum provided a significant protective effect when compared to blank skin postapplication of lipid extractor.

71.2.2 *In Vivo* Methods

Mahmound and Lachapelle (1985) and Lachapelle et al. (1990) developed a guinea pig model to evaluate the protective value of BC and gels by laser Doppler flowmetry (blood flow) and histological assessment. The histopathological damage after 10 min of toluene contact was mainly confined to the epidermis. Dermal blood flow changes were relatively high on the control site compared to the gel pretreated sites.

Frosch et al. (1993b–d, 1994) established the repetitive irritation test (RIT) in guinea pig and in humans to evaluate the efficacy of BC using series of bioengineering techniques. The cream pretreated and untreated test skin (guinea pig or humans) were exposed daily to the irritants for 2 weeks. The resulting irritation was scored on a visual scale and assessed by parameters of biophysical (bioengineering) techniques. Some test creams suppressed irritation with all test parameters; some even increased irritation.

Fullerton and Menne (1995) performed an *in vivo* patch testing with nickel-sensitive patients by using nickel disks with and without barrier gels. Test preparations and nickel disks were removed 1-day post application, and the test sites evaluated. Barrier gel treated sites significantly reduced the positive test reactions.

Grunewald et al. (1995) utilized a SLS repetitive washing model to evaluate the protective effects of BC by measuring with bioengineering techniques on 15 human volunteers. All BC reduced the deterioration of skin functions following 1 week repetitive washing. Subsequently, they also found urea- and glycerol oil-in-water emulsions provided a greater protection against a lipophilic irritant (toluene) after 7 days of repetitive irritation (Grunewald et al., 1996).

Marks et al. (1995) investigated a topical lotion containing 5% Q18B in the prevention of experimentally induced poison ivy and poison oak ACD in susceptible volunteers. One hour before both forearms were patch tested with urushiol, 5% Q18B lotion was applied on one forearm. The test patches were removed after 4 h and the sites interpreted for reaction 2, 5, and 8 days later. The test sites pretreated with Q18B lotion had nill or significantly reduced reactions to the urushiol compared with untreated control sites ($p < .0001$) on all test days.

Zhai and Maibach (1996b) measured the effectiveness of BC in an *in vivo* human model against dye indicator solutions: methylene blue in water and oil red O in ethanol, representative of model hydrophilic and lipophilic compounds. Solutions of 5% methylene blue and 5% oil red O were applied to untreated and BC pretreated skin with the aid of aluminum occlusive chambers, for 0 h and 4 h. Post application time, materials were removed, and consecutive skin surface biopsies obtained. The amount of dye penetrating into each strip was determined by colorimetry. Two model creams exhibited effectiveness, but one enhanced the cumulated amount of dye.

Schlüter-Wigger and Elsner (1996) assessed four commercially available BC against four standard irritants: 10% SLS, 1% sodium hydroxide (NaOH), 30% lactic acid (LA), and undiluted toluene (TOL) in the RIT in humans for 12 days. Irritation was assessed by visual scoring, transepidermal water loss (TEWL), and colorimetry. All products were very effective against SLS irritation. No BC provided significant protection against TOL. Three products showed a partially protective effect against all ionic irritants, while the fourth showed less protection against SLS and NaOH, and even amplification of inflammation by TOL. Wigger-Alberti and Elsner (1997) evaluated petrolatum utilizing the above model; petrolatum was effective against SLS, NaOH, and LA irritation, and provided a moderate protection against TOL. Wigger-Alberti et al. (1998) also examined three other BC and petrolatum against 10% SLS, 0.5% NaOH, 15% LA, and undiluted TOL in the RIT in humans for 9 days. All BC exhibited a significant protective effect against irritation by SLS, NaOH, and LA. Less efficacy was observed against TOL. In another 12-days RIT study (Wigger-Alberti et al., 1999), white petrolatum provided a significant protective effect

against SLS, NaOH, and TOL but with less protective effect against LA irritation.

de Fine Olivarius et al. (1996) determined BC efficacy in protecting against water, based on evaluation of color intensities when an aqueous solution of crystal violet is applied to the skin, after pretreatment with different creams. The BC with particles gave the best immediate protection (dorsal 76%, volar 69%). The moisturizer was intermediately protective (dorsal 57%, volar 34%), while little protection was found for the silicone-containing cream (dorsal 16%, volar 10%).

Fartasch et al. (1998) investigated protective capacity of a lipophilic BC on acute ICD by TEWL measurement. Application of the BC before and during irritation showed a decrease of TEWL by 58% (back) and 49% (arm).

Elsner et al. (1998) evaluated perfluoropolyethers (PFPE) containing BC against a set of four irritants: 10% SLS, 0.5% NaOH, 15% LA, and undiluted TOL in the RIT on the human back. Irritation was assessed by visual scoring, TEWL, and colorimetry. All PFPE preparations significantly suppressed irritation by SLS and NaOH. However, only the 4% PFPE preparation was significant against LA and TOL.

Zhai et al. (1998) introduced a facile approach to screening protectants *in vivo* in human subjects. Two acute irritants and one allergen were selected: SLS, the combination of ammonium hydroxide (NH_4OH) and urea, and Rhus. The model irritants and allergen were applied with an occlusive patch for 24 h. Inflammation was scored with an expanded 10 point scale at 72 h postapplication. Most test protectants statistically suppressed SLS irritation and Rhus allergic reaction but not NH_4OH and urea induced irritation. They further utilized this model to evaluate the putative skin-protective formulations (Zhai et al., 1999b). All formulations failed to inhibit NH_4OH and urea irritation. Only paraffin wax in cetyl alcohol statistically ($p < .01$) reduced Rhus-ACD. Three commercial formulations markedly ($p < .001$) suppressed SLS-ICD.

Shimizu and Maibach (1999) used squamometry method to evaluate a barrier protectant (tannic acid). Five percent tannic acid and distilled water (as a control) were applied to forearms for 30 min; these pretreated sites were dosed with different concentrations of SLS for 24 h. Squamometric evaluation indicated that skin damage increased with SLS concentration in a dose-dependent manner, and tannic acid significantly reduced the damage ($p < .01$).

Vidmar and Iwane (1999) assessed the ability of the topical skin protectant (TSP) to protect against urushiol (Rhus)-ACD. Open urushiol patch testing was conducted on 50 rhus-sensitive subjects. After 96 h, dermatitis severity scores were compared between TSP protected and TSP unprotected sites by using a nine-point dermatitis scale. Results showed that TSP protected sites had mean dermatitis scores about two points lower than TSP unprotected sites ($p < .001$).

Patterson et al. (1999) determined the preventive effect of a skin protectant containing dimethicone and glycerin with various inactive ingredients in an aerosol foam against SLS-ICD and poison ivy and poison oak (urushiol)-ACD. Skin reaction was assessed periodically for 10 days by using a 0–7 point dermatitis scale. The formulation was significantly effective in reducing SLS irritation but did not prevent urushiol-ACD.

Zhai et al. (2000) evaluated the efficacy of a dimethicone skin protectant lotion against SLS-ICD by clinical visual grading and bioengineering techniques in humans. Both forearms were pretreated either with the testing protectant lotion or with its vehicle control prior to contact with SLS. Thirty minutes later, 0.5% SLS was applied to each pretreated site for 24 h. One additional site received SLS only. The efficacy of the protective effect was determined by visual scoring (VS), TEWL, skin color (a^* value), and cutaneous blood flow volume (BFV). VS and TEWL data showed a significant decrease on the protectant lotion pretreated site in comparison with SLS only treated site as well as the vehicle control site. But, BFV and a^* values did not show a statistical difference between either treated sites.

Berndt et al. (2000) investigated the efficacy of a BC and its vehicle in a field setting: two panels of 25 hospital nurses with mild signs of skin irritation were asked to use one of the test products (BC or its vehicle) and especially to use it before contact with skin irritants over 4 weeks. Effects of both preparations were studied weekly by clinical examination and bioengineering measurements. Results showed no significant differences between BC and its vehicle. In both groups, clinical skin status improved and stratum corneum hydration increased significantly. They concluded that the vehicle alone is capable of positively influencing skin status.

Schnetz et al. (2000) introduced a standardized test procedure for the evaluation of skin protective products. A ROIT with a standardized protocol has been evaluated in two phases (12 days and 5 days protocol) in several clinical centers. Skin was treated by two irritants (0.5% SLS and toluene, twice a day for 30 min). Inflammation was measured by bioengineering methods (TEWL and colorimetry) and clinical scoring. The 5-day protocol was sufficient to achieve significant results. Furthermore, in spite of the expected inter-center variations due to heterogeneity of the individual threshold of irritation, interpretation of clinical score, and inter-instrumental variability, the ranking of the vehicles regarding reduction of the irritant reaction was consistent in all centers.

McCormick et al. (2000) measured the efficacy of a BC and an oil-containing lotion for protecting the hands of health care workers with severe hand irritation. Objective and subjective parameters for scaling, cracking, weeping, bleeding, and pain were scored by two blinded investigators weekly for 4 weeks. Subjects in both groups experienced marked improvement in overall hand condition (each, $p < .02$), particularly in scaling, cracking, and pain. Volunteers randomized to use the oil-containing lotion showed the greatest improvement.

Sun et al. (2000) utilized laser-induced breakdown spectroscopy (LIBS) to evaluate the effect of BC on human skin; three representatives of commercial BC advertised as being effective against lipophilic and hydrophilic

TABLE 71.1
BC Efficacy and Testing Models from Recent Experiments

Models		Irritants or Allergens	BC	Efficacy	Authors and References
In Vitro	*In Vivo*				
Human skin		Dyes (eosin, methylviolet, oil red O)	16 BC	Protection varied	Treffel et al. (1994)
Human skin	Nickel-sensitive patients	Nickel disk	Ethylenediamine tetra acetic acid (EDTA) gels	Significantly reduced the amount of nickel in the epidermis *in vitro*, and significantly reduced positive reactions *in vivo*	Fullerton and Menne (1995)
Thin protective product film	Humans	Moisture penetration	Petrolatum and a protectant	Petrolatum offered some protection against water penetration and a hydroactive polymer system (protectant) prevented moisture penetration	Shah and Kirchner (1997)
Human skin		SLS and a mixture of hexane and methanol	Six products	Petrolatum exhibited the best protective effect against SLS and also provided good protection to organic solvents	Goffin et al. (1998)
Human skin		[^{35}S]-SLS	Three Q18B gels	Protection effect (%) was 88%, 81%, and 65%, respectively	Zhai et al. (1999a)
Fresh and frozen human skin		Tritiated water	Two BC	Both BC lowered the average flux rates of tritiated water through fresh and frozen skin	van Der Bijl et al. (2000)
	Guinea pig	TOL, n-hexane, and trichlorethylene	Antisolvent gel and other three BC	Dermal blood flow changes were relatively high on the control site compared to the gel pretreated sites. They found that BC can protect against TOL, n-hexane but not trichlorethylene	Mahmound and Lachapelle (1985); Lachapelle et al. (1990)
	Guinea pigs and humans	SLS, sodium hydroxide, TOL, and LA	Several BC	Some suppressed irritation, some failed, and some exacerbated	Frosch et al. (1993b,c,d, 1994)
	Humans	TOL	Several BC	All tested BC markedly reduced the irritating effect of repetitive toluene contact	Grunewald et al. (1995)
	Humans with a history of allergy to poison ivy/oak	Urushiol	Q18B lotion	Q-18B lotion significantly reduced reactions to the urushiol	Marks et al. (1995)
	Humans	Dyes (methylene blue and oil red O)	Three BC	Two exhibited effectiveness; one enhanced cumulative amount of dye	Zhai and Maibach (1996b)
	Humans	10% SLS, 1% NaOH, 30% LA, and TOL	Four BC and white petrolatum	Different protective effects were detectable. All products were effective against SLS irritation	Schlüter-Wigger and Elsner (1996)
	Humans	Water	Two BC and a moisturizer	BC with particles provided the greatest immediate protection (dorsal 76%, volar 69%)	de Fine Olivarius et al. (1996)
	Humans	SLS	A lipophilic BC	A decrease of TEWL by 58% (back) and 49% (arm)	Fartasch et al. (1998)
	Humans	SLS, NaOH, LA, and TOL	BC containing PFPE	All BC significantly suppressed irritation by SLS and NaOH. Only the 4% PFPE-containing preparation was significant against LA and TOL	Elsner et al. (1998)
	Humans	SLS, NH_4OH and urea, Rhus	Several protectants	Most suppressed SLS irritation and Rhus ACD, but failed to suppress NH_4OH and urea irritation	Zhai et al. (1998)
	Humans	SLS	A barrier protectant (tannic acid)	Tannic acid significantly reduced SLS induced damage	Shimizu and Maibach (1999)

	Rhus-sensitive subjects	Urushiol	TSP	TSP protected sites had lower dermatitis scores than TSP unprotected sites	Vidmar and Iwane (1999)
	Humans and Rhus-sensitive subjects	SLS-ICD and poison ivy and poison oak (urushiol)-	A formulation containing dimethicone and glycerine	Test formulation significantly effective in reducing the irritation from SLS but did not prevent urushiol-ACD	Patterson et al. (1999)
	Humans	SLS	A dimethicone containing skin protectant lotion	VS and TEWL data showed a significant decrease on the protectant lotion pretreated site in comparison with SLS only treated site as well as vehicle control site	Zhai et al. (2000)
	Nurses with mild signs of skin irritation	Occupational risk exposures	A test BC or its vehicle	No significant differences between BC and its vehicle. In both groups, clinical skin status improved and stratum corneum hydration increased significantly during the study	Berndt et al. (2000)
	Humans	SLS and TOL	Three BC	Various protective effects were detectable with 5-day study protocol to achieve significant results	Schnetz et al. (2000)
	Health care workers with severe hand irritation	Working environments	One BC and an oil-containing lotion	Subjects in both groups experienced marked improvement in overall hand condition, but use of the oil-containing lotion showed greater improvement	McCormick et al. (2000)
	Humans	Zinc	Three representative commercial BC	BC provided appreciable protection against the penetration of both $ZnCl_2$ and ZnO into skin	Sun et al. (2000)
	Humans	Two types of latex gloves	A test BC	BC decreased latex gloves-induced contact urticaria syndrome	Allmers (2001)
	Humans	Latex glove	A model lipid emulsion	The test emulsion minimized glove induced-ICD.	Zhai et al. (2002)
Three-dimensional skin culture		SDS	One water-in-oil emulsion, petrolatum, and one water-in-oil-water emulsion	The multiple emulsion reduces cell damage after treatment with SDS	zur Muhlen et al. (2004)
	Humans	SLS	A containing 5% dexpanthenol formulation	A formulation containing 5% Dexpanthenol exhibits protective effects against skin irritation	Biro et al. (2003)
	Humans	SDS	One water-in-oil emulsion, petrolatum, and one water-in-oil-water emulsion	The multiple emulsion provided the best protection against SDS	zur Muhlen et al. (2004)
	Volunteers who were hypersensitivity to latex gloves	Latex gloves; SLS	A topical formulation containing a zinc gel	The zinc gel significantly reduced skin irritation when exposed to latex gloves. In addition, it also prevented dermal irritation induced by SLS	Modak et al. (2005)
Human cadaver skin		Lipid extractor: [Chloroform: methanol (2:1)]	Five skin protective agents /formulations	Only petrolatum showed a significant protective effect when compared to blank skin	Barbadillo et al. (2006)

substances were evaluated by measuring the zinc absorbed through the stratum corneum. Four consecutive skin surface biopsis (SSBs) were taken from biceps of the forearms of six volunteers at time periods of 0.5 h and 3 h after BC application. The BC provided appreciable protection against the penetration of both $ZnCl_2$ and ZnO into the skin when compared with control skin (without BC treated).

Allmers (2001) tested two types of latex gloves with and without the use of a BC on subjects who had type I hypersensitivity reactions to natural rubber latex gloves. One hand received BC for 10 min, before both hands utilized gloves for 30 min. BC decreased latex gloves-induced contact urticaria syndrome.

Zhai et al. (2002) evaluated the prevention of wearing occlusive glove-induced ICD by a model lipid emulsion. Test emulsion was applied to one hand, while the opposite hand remained untreated. Thirty minutes later, both hands were gloved for 3 h. Skin conditions were evaluated by visual scoring, water sorption-desorption test, TEWL, and skin capacitance. This procedure was repeated for 5 days. Emulsion treated hands showed significantly greater water holding capacity and lower TEWL values when compared to untreated hands. They concluded that the test emulsion minimized glove induced-ICD.

Biro et al. (2003) investigated the efficacy of dexpanthenol in skin protection against irritation in a randomized, prospective, double-blind, placebo-controlled study. Twenty-five healthy volunteers were treated on the inner aspect of both forearms with a containing 5% dexpanthenol or placebo twice daily for 26 days. From day 15 to 22, 2% SLS was applied to these areas twice daily. Documentation comprised sebumetry, corneometry, pH value, and clinical appearance (photographs). They concluded that with a solution containing 5% dexpanthenol exhibits protective effects against skin irritation.

Zur Muhlen et al. (2004) assessed the efficacy of three products by an *in vivo* ROIT method.

They demonstrated that a multiple emulsion provided the best protection against SDS. They believed that the multiple emulsion increased the content of skin lipids, which reduced the inducing irritation or cell death caused by SDS.

Modak et al. (2005) evaluated a topical formulation containing a zinc gel as a prophylactic against latex glove-related contact dermatitis. Twenty-two volunteers who exhibited mild to moderate contact dermatitis (type IV) after wearing latex gloves were included in this study. Results indicated that the topical formulation containing a zinc gel significantly reduced skin irritation in volunteers who exhibited type IV hypersensitivity when exposed to latex gloves. In addition, it also prevented dermal irritation induced by SLS.

Table 71.1 summarizes the recent experimental models and BC efficacy.

71.3 CONCLUSIONS

BC have shown the efficacy to diminish the development of CD from *in vitro* and *in vivo* methods described in this chapter. However, their actual benefits should be evaluated in the workplace as a supplement to model experiments. Additionally, many factors may influence the actual effectiveness of the BC (Packham et al., 1994; Wigger-Alberti et al., 1997a,b).

These *in vitro* and *in vivo* models have been well developed; they provide insight into mechanisms as well as greater discriminatory potential. *In vitro* models are widely used to test the effects of BC because they are simple, rapid, and safe. In particular, they are recommended as a screening procedure for BC candidates. Radiolabeled methods may determine the accurate protective and penetration results even with lower levels of chemicals due to the sensitivity of radiolabeled counting. Animal experiment may be used to generate kinetic data because of a closer similarity between humans and some animals (pigs and monkeys) in percutaneous absorption and penetration for some compounds. But no one animal, with its complex anatomy and biology, will simulate penetration in humans for all compounds. Therefore, the best estimate of human percutaneous absorption is determined by *in vivo* studies in humans. Histological assessments may define what layers of skin are damaged or protected, and may provide insight in BC mechanisms. Noninvasive bioengineering techniques provide accurate, highly reproducible, and objective observations in quantifying the inflammation response to various irritants and allergens; they can assess subtle differences to supplement traditional clinical studies.

Though BC may act against many irritants or allergens, their benefits are individual, i.e., to specific kinds of chemical. Regarding testing method, there is no standardized procedure so far. Taken together, these models accelerate product development because of their efficiency and simplicity. Well-controlled intervention studies will, in the end, lead to greater BC widespread utilization.

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72 Light-Induced Dermal Toxicity: Effects on Cellular and Molecular Levels

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72.1 INTRODUCTION

> Die Sonne ist auch da wenn die Wolken schwarz und undurchdringlich scheinen.
>
> **Ernst Jucker (1957)**[*]

Toxicology has evolved as a multidisciplinary field of study and is still in rapid evolutionary development. As such, toxicology overlaps many other basic biomedical disciplines, including biochemistry, pharmacology, and physiology. A recent event in this development has been the intersection of toxicology with photobiology, opening the field of phototoxicology.

Sunlight is the most potent environmental agent influencing life on the earth. Historically, exposure to the sun has been believed to be healthful and beneficial. It has only recently become apparent that many of the effects of solar radiation are detrimental. In a broad sense, therefore, the evolution of life can be regarded a continuous adaptation to light by simultaneously utilizing solar energy and protecting against its detrimental effects.

Modern civilization presents a challenge for basic phototoxicologic research. This challenge arises from alterations in the life-styles of a large portion of the population, including holiday trips, clothing styles, and particularly the fashion of suntanning among Caucasians. It is also possible that environmental factors may change the spectral characteristics of light reaching the earth's surface. Many of these factors lead to an essentially increased exposure to light for a large segment of the population (Fitzpatrick et al., 1974; Urbach, 1989). Furthermore, in the past decade, phototoxic reactions to drugs, cosmetics, and many industrial and environmental chemicals have become an important health problem.

Definitions of phototoxicity are numerous and frequently inconsistent. In the broadest sense, any toxicity induced by photons can be termed photosensitivity. Photosensitivity may involve either photoallergies or nonimmunologic photoinduced skin reactions. Phototoxicity is used to describe all nonimmunologic light-induced toxic skin reactions. Sunburn is the most frequently occurring phototoxic reaction, requiring only the interaction of ultraviolet (UV) light with skin. In most cases of phototoxicity, however, we deal with an endogenous or exogenous chemical (chromophore) that absorbs light and transfers the energy to, or reacts in the excited state with, cellular components. Such toxic reactions would most properly be termed chemical phototoxicity.

Chronic phototoxic exposure can lead to neoplastic changes. It is established that the consequence of lifelong enhanced exposure to light is a significant increase in skin

[*] "The sun is still present even when the clouds seem dark and impenetrable." From *Ein gutes Wort zur rechten Zeit*, Bern: Verlag Paul Haupt.

tumors (Urbach et al., 1974), including basal and squamous cell carcinomas and, to a certain extent, malignant melanomas. This is confirmed by the pronounced increase in frequency of skin cancers in that part of the population, particularly those Celts and Teutons, that in the course of history settled in regions with higher solar irradiation (Africa, Australia, and North America).

Phototoxicity studies, particularly those related to human disorders, have so far been based predominantly on gross anatomic or histological procedures. Although our knowledge of the molecular events that occur during these processes is rapidly growing, much basic research remains to be done. In this chapter, we discuss some molecular and cellular events that take place on exposure to light.

72.2 LIGHT CHARACTERISTICS

Aside from artificial light sources, solar radiation is the primary source of light that elicits biological effects. A portion of the solar spectrum containing the biologically most active region (290–700 nm) is shown in Figure 72.1.

The UV part of the spectrum includes wavelengths from 200 to 400 nm. Portions of the UV spectrum have distinctive features from both the physical and medical points of view. The accepted designations for the biologically important parts of the UV spectrum are UVA, 320–400 nm; UVB, 290–320 nm; and UVC, 220–290 nm (Figure 72.2).

Wavelengths less than 290 nm (UVC) do not occur at the earth's surface, since they are absorbed, predominantly by ozone, in the stratosphere. The most thoroughly studied photobiological reactions that occur in skin are induced by UVB. Although UVB wavelengths represent only approximately 1.5% of the solar energy received at the earth's surface (World Health Organization, 1979), they elicit most of the known biological effects. Light distributed over these wavelengths inhibits cell mitosis; makes vitamin D; and induces sunburn, skin aging, and skin cancer. The UVA region elicits most of the known chemical phototoxic and photoallergic reactions. It has been proposed that the longer wavelengths of the UVA spectrum (UVA I: 340–400 nm) are less detrimental than the shorter UVA wavelengths (UVA II: 320–340 nm) (National Institutes of Health Consensus Development Conference Statement, 1989). Recent studies conducted on the influence of UVA on biological systems show that a great variety of effects are induced by these wavelengths. The findings of these studies have become increasingly important because of the popularity of indoor tanning, which can involve high UVA doses from powerful UVA sources. Research has shown that within the UVA spectrum lies the peak response for immediate pigment darkening (Irwin et al., 1993). Skin cancer, once thought to be induced solely by UVB, also results from UVA exposure (Sterenborg and Van Der Leun, 1990). In addition, protein kinase C, which has been linked to chemical tumor promotion, and may play a role in UV-induced tumor promotion, was shown to be induced by UVA in cultured mouse fibroblasts (Matsui and DeLeo, 1990). Similarities have been found between UVA and ionizing radiation with respect to DNA damage induction. Unlike single-strand breaks, which are efficiently repaired by normal cells, DNA double-strand breaks are thought to be a critical lethal lesion. UVA, as well as UVB, UVC, and ionizing radiation has

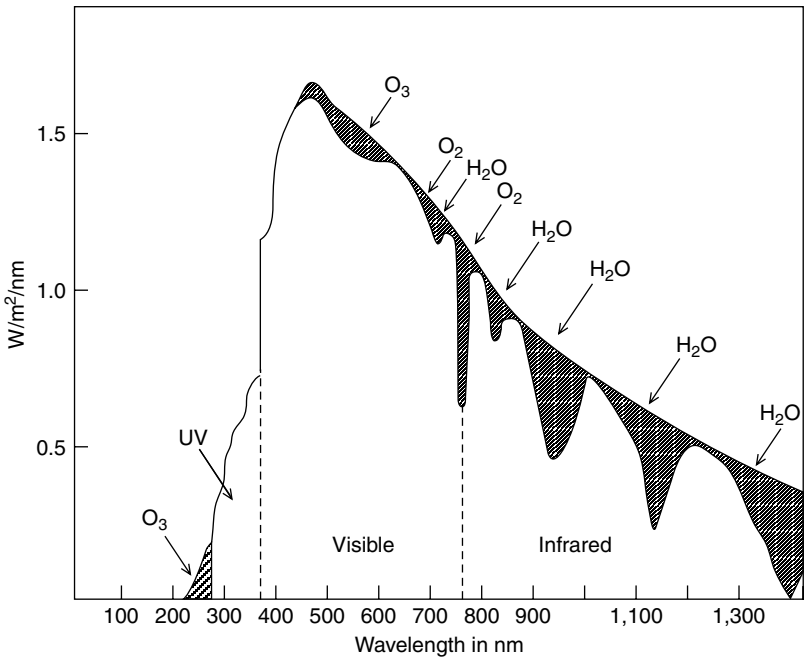


FIGURE 72.1 Spectrum of solar energy received at the earth's surface. The absorption bands of atmospheric O_2, O_3, and H_2O are shown. (Modified from Hynek, J.A., *Astrophysics: A Topical Symposium Commemorating the Fiftieth Anniversary of the Yerkes Observatory and a Half Century of Progress in Astrophysics*, McGraw-Hill, New York, 1951.)

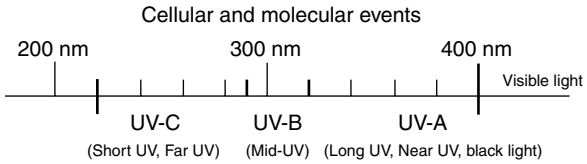


FIGURE 72.2 The UV regions of the solar spectrum.

been shown to induce double-strand breaks in DNA of cultured human epithelioid cells (Peak and Peak, 1990). UVA, not UVB, caused mouse skin to become highly resistant to solubilization by pepsin digestion, possibly as a result of increased cross-linking of dermal collagen (Kligman and Gebre, 1991). UVA effects not shared with UVB or UVC have been reported for nonnuclear damage and cell lysis in three strains of murine lymphoma cell lines (Beer et al., 1993) and for the oxidation of cytoplasmic components causing adverse cytoskeleton effects resulting in hemolysis of sheep red blood cells (Godar et al., 1993).

The visible portion of the spectrum, representing about 50% of the sun's energy received at sea level, includes wavelengths from 400 to 700 nm. Visible light is necessary for such biological events as photosynthesis, circadian cycles, and vision. Furthermore, visible light in conjunction with certain chromophores (e.g., dyes, drugs, and endogenous compounds) and molecular oxygen induces photodynamic effects.

Understanding the toxic effects of light impinging on the skin requires knowledge of the skin's optical properties. Skin may be viewed as an optically nonhomogeneous medium, composed of three layers that have characteristic refractive indices, chromophore distributions, and light-scattering properties. Light of wavelengths between 250 and 3000 nm entering the outermost layer of the skin, the stratum corneum, is in part reflected approximately 4–7% due to the difference in refractive index between air and stratum corneum (Fresnel reflection) (Anderson and Parrish, 1981).

Absorption by urocanic acid (a deamination product of histidine), melanin, and proteins containing the aromatic amino acids tryptophan and tyrosine in the stratum corneum produces further attenuation of light, particularly at shorter UV wavelengths. Approximately 40% of the UVB is transmitted through the stratum corneum to the viable epidermis (Everett et al., 1966). The light entering the epidermis is attenuated by scattering and, predominantly, absorption. Epidermal chromophores consist of proteins, urocanic acid, nucleic acids, and melanin. Passage through the epidermis results in appreciable attenuation of UVA and particularly UVB radiation. The transmission properties of the dermis are largely due to scattering, with significant absorption of visible light by melanin, carotenoids, and blood-borne pigments such as bilirubin, hemoglobin, and oxyhemoglobin. Light traversing these layers of the skin is extensively attenuated, most drastically for wavelengths less than 400 nm. Longer wavelengths are more penetrating. It has been noted that there is an "optical window," that is, greater transmission for light at wavelengths of 600–1300 nm, which may have important biological consequences (Anderson and Parrish, 1981). These features are presented in Figure 72.3.

Normal variations in the skin's melanin content may result in changes in the attenuation of light, particularly in those wavelengths between 300 and 400 nm, by as much as 1.5 times more in Negroes than in Caucasians (Pathak, 1967). Alterations in the amount or distribution of other natural chromophores account for further variations in the skin's optical properties.

Urocanic acid deposited on the skin's surface during perspiration (Anderson and Parrish, 1981) and UV-absorbing lipids excreted in sebum (Beadle and Burton, 1981) may significantly reduce UV transmission through the skin. Epidermal thickness, which varies over regions of the body and increases after exposure to UVB radiation, may significantly modify UV transmission (Soffen and Blum, 1961; Parrish and Jaenicke, 1981).

Certain disease states also produce alterations in the skin's optical properties. Alteration of the skin's surface, such as by psoriatic plaques, decreases transmitted light. This effect may be lessened by application of oils whose refractive index is similar to that of skin (Anderson and Parrish, 1981). Disorders such as hyperbilirubinemia, porphyrias, and blue skin nevi result in increased absorption of visible light due to accumulation or altered distribution of chromophoric endogenous compounds.

The penetration of light into and through dermal tissues has important consequences. Skin, as the primary organ responsible for thermal regulation, is overperfused with blood relative to its metabolic requirements (Anderson and Parrish, 1981). It is estimated that the average cutaneous blood flow is 20–30 times that necessary to support the skin's metabolic needs. The papillary boundaries between epidermis and dermis allow capillary vessels to lie close to the skin's surface, permitting the blood and important components of the immune system to be exposed to light. The equivalent of the entire blood volume of an adult may pass through the skin, and potentially be irradiated, in 20 min. This corresponds to the time required to receive one to two minimal erythema doses (MEDs).* The accessibility of incident radiation to blood has been exploited in such regimens as phototherapy of hyperbilirubinemia in neonates, where light is used as a therapeutic agent. However, in general there is a potential for light-induced toxicity due to irradiation of blood-borne drugs and metabolites.

Of course light-induced damage is not confined to the skin. Ocular injury and aging of the eye can result from oxidative stress in tissues caused by radiant energy. Owing to various molecular species in the eye, different parts of the eye absorb different wavelengths; the cornea absorbs UVB, the lens absorbs the majority of UVA and some UVB, and the retina and pigment epithelium absorb all the blue light (Zigman, 1993).

* The minimal erythema dose (MED) is defined as the minimal dose of UV radiation that produces definite, but minimally perceptible, redness 24 h after exposure.

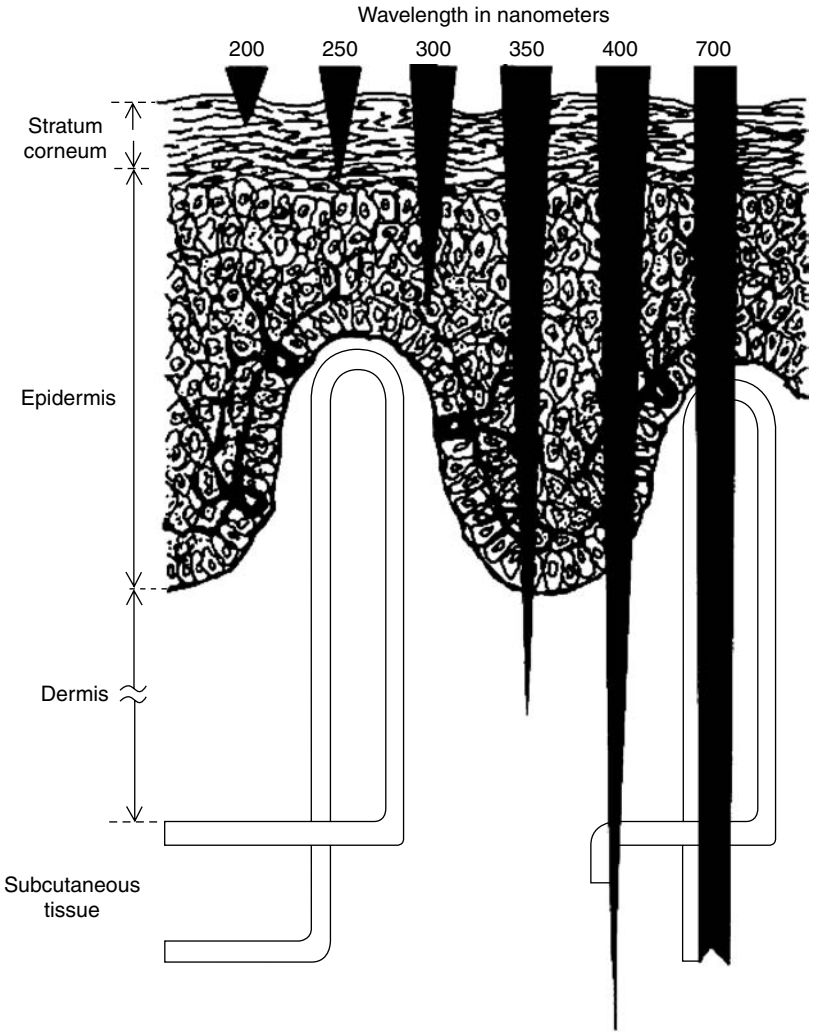


FIGURE 72.3 Schematic representation of light penetration into skin.

72.3 FUNDAMENTAL CONCEPTS IN PHOTOCHEMISTRY

Damage to cells through a photoreaction is initiated at the site where the chromophore absorbs specific wavelengths of light. Absorption of UV or visible photons results in electronically excited molecules; dissipation of this energy may result in an adverse phototoxic effect on the cell. The sequence of events initiated by light absorption is shown in Figure 72.4.

The transition of a ground-state molecule to an excited singlet electronic state accompanies absorption of a visible or UV photon. Molecules in their singlet excited states exist for only about 10^{-8} to 10^{-9} s before either returning to the ground state or converting (intersystem crossing) to a long-lived (10^{-4} to 10^{1} s) metastable triplet state. Both excited singlet and triplet states relax to the ground state through (1) transfer of energy to another molecule and (2) emission of light (fluorescence or phosphorescence) or release of heat.

Alternatively, the excited molecule may undergo photochemistry such as *cis–trans* isomerization, fragmentation, ionization, rearrangement, and intermolecular reactions. The probability that an excited molecule will choose any given path to the ground state depends on both its molecular structure

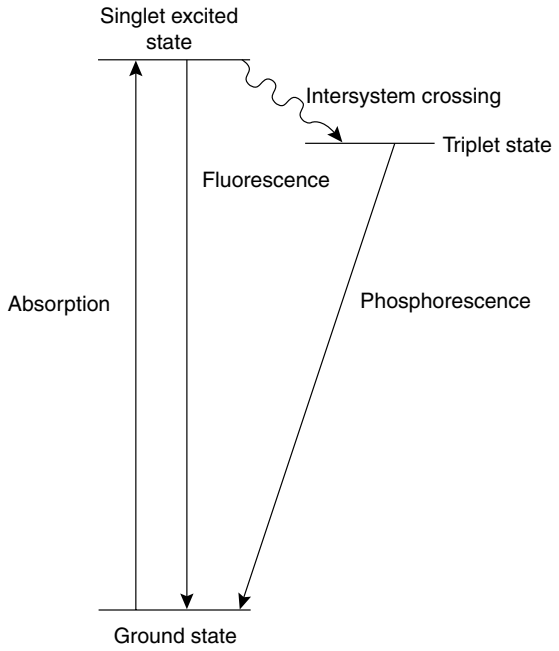


FIGURE 72.4 Electronic energy diagram of physical events accompanying the absorption of a photon.

and its environment and may be determined experimentally (Turro, 1965).

All these factors, such as light absorption, the nature of the excited states, the extent of intersystem crossing, and photochemical reactions, will finally determine the phototoxic potential of an endogenous or exogenous compound. However, we are not yet able to predict the phototoxic potential of a compound from its molecular structure alone. Reliable predictive tests are still required to evaluate suspected compounds. Several lists of compounds that are phototoxic in humans have appeared (e.g., Parrish et al., 1979). Classes of compounds known to be phototoxic in humans are

Psoralens	Sulfonamides
Sulfonylureas	Phenothiazine
Tetracyclines	Coal tar
Anthracene	Acridine
Phenanthrene	Fluoroquinolones

The mechanisms through which absorption of light causes a chemical alteration in the chromophore, eventually resulting in a phototoxic response, are shown in Figure 72.5.

Compounds such as psoralens may react directly in their excited states with a biological target. Because of the short lifetimes of most excited states, direct reactions require close association, or complex formation, between the chromophore and the target before light absorption. Alternatively, a stable toxic photoproduct may be formed after absorption of light. Chlorpromazine and protriptyline are examples of this mechanism (Kochevar, 1981). The phototoxicity of these compounds is in large part the result of the toxicity of their photoproducts.

The other mechanisms shown in Figure 72.5 are frequently categorized as photodynamic mechanisms. Photodynamic reactions usually involve compounds that absorb UVA or visible light. In type I photodynamic reactions, the chromophore in an excited triplet state is reduced either by an electron or by hydrogen transfer from a compound in the environment. This reduction results in the

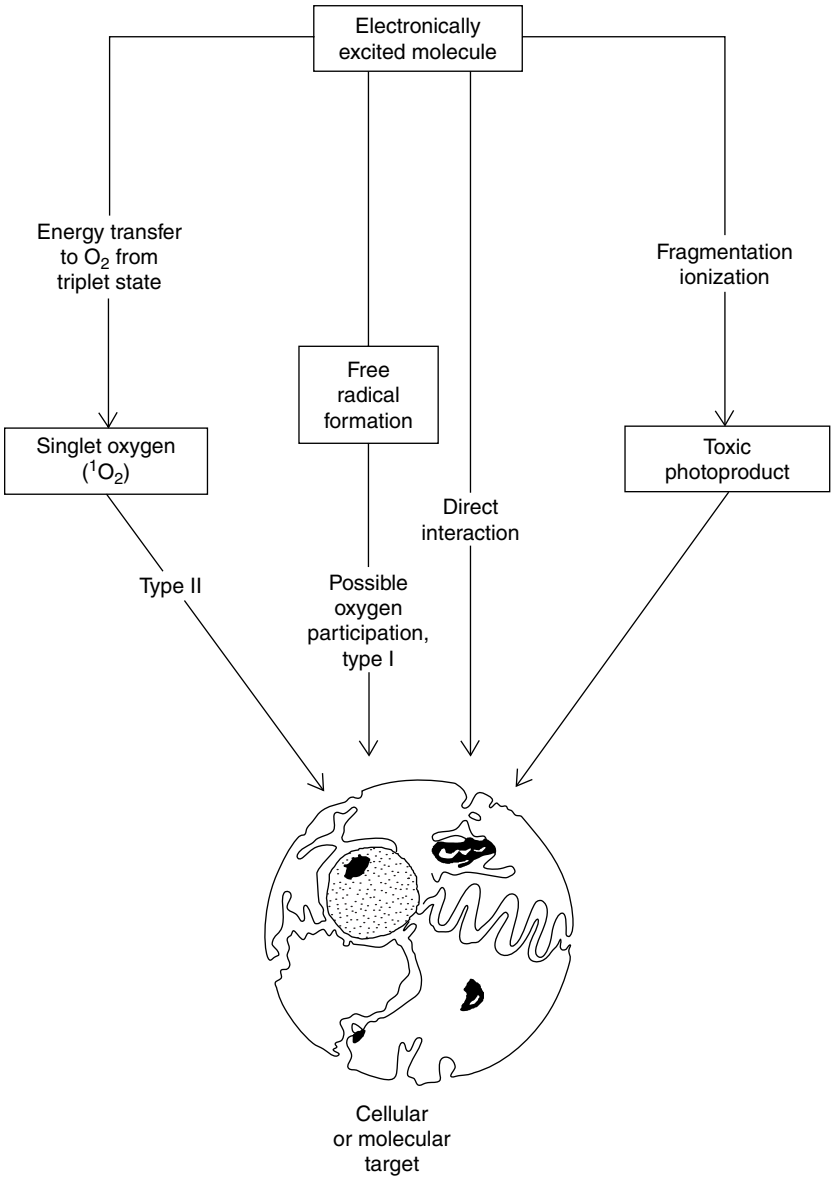


FIGURE 72.5 Diagram of basic phototoxicity mechanisms. The electronically excited molecule, located within or near a cell, may elicit a phototoxic response through several mechanisms.

generation of highly reactive free radicals, whose subsequent attack on biological substrates may result in toxicity. In type II photodynamic reactions, the chromophore transfers its energy to O_2, generating singlet oxygen ($^1O_2$), an active oxidizing agent. A large body of evidence now supports the involvement of $^1O_2$ in photodynamic reactions. Recently, investigators have demonstrated photosensitized formation of $^1O_2$ in *in vitro* as well as *in vivo* studies (Baker and Kanofsky, 1991, 1993; Oelckers et al., 1999; Niedre et al., 2002).

72.4 CELLULAR TARGETS AND MECHANISMS OF PHOTOTOXICITY

A vigorous effort is under way to discover the biological targets in phototoxicity. Cellular injury by photons may be studied on either the histological or the molecular level. The characteristic histological change induced by photons is the appearance of the so-called sunburn cell (SBC) (Daniels et al., 1961), a dyskeratotic cell with bright eosinophilic cytoplasm and a pyknotic nucleus. SBCs appear 24–48 h after UVB irradiation (Woodcock and Magnus, 1976) and may persist 1 week or longer (Parrish et al., 1979). The mechanisms of SBC formation are still obscure, although its morphological and biochemical characteristics have been investigated (Danno and Horio, 1980; Olson et al., 1974). The primary chromophore for sunburn cell production is not known; however, Young and Magnus (1981) found evidence that DNA may be an important chromophore. They detected SBCs in mouse epidermis after administration of 8-methoxypsoralen (8-MOP) followed by UVA irradiation (psoralen + UVA is abbreviated as PUVA). They speculated that since the primary molecular lesion in PUVA treatment is in DNA, the fact that PUVA can promote SBC formation supports the view that DNA may be a significant chromophore in SBC induction.

The mechanisms by which photosensitized cells are damaged are in most cases poorly understood. On the subcellular level, the primary targets in a phototoxic reaction include nucleic acids, proteins, and plasma and organelle membranes. Subcellular effects may differ depending on the photosensitizer's structure and intracellular localization. Sensitizers such as rose bengal, porphyrin, and anthracene accumulate selectively in cell plasma membranes (Ito, 1978). Acridine orange and psoralens accumulate in the cell nucleus (Van de Vorst and Lion, 1976; Pathak et al., 1974; Bredberg et al., 1977). Recently, it was reported that psoralens also accumulate in cell membranes. These membrane-bound psoralens may initiate important biological effects (Laskin et al., 1985). Some photosensitizers may become concentrated in lysosomes and on irradiation may induce lysosomal rupture (Allison et al., 1966). Table 72.1 shows results from some studies of the mechanisms of action for several important classes of phototoxic compounds.

It includes two endogenous photosensitizers, porphyrins, and kynurenic acid. As reflected in Table 72.1, most compounds that evoke chemical phototoxicity are thought to act through a photodynamic mechanism. Further, it appears that a compound may elicit a phototoxic response through several modes. Studies are needed to correlate specific molecular alterations (such as DNA cross-linking and photooxidation of enzymes and of DNA) with cell toxicity and mutagenesis. To date, the mechanism of psoralen phototoxicity is relatively well understood. Much more remains to be learned about the mode of action for other groups of photosensitizers.

72.5 SPECIFIC MOLECULAR ALTERATIONS IN CELLS

On the molecular level, DNA is the most critical target in a cell exposed to UV light. As previously discussed, other cellular constituents may also be affected, generally with less severe consequences for the cell.

72.5.1 Thymine Photoproducts

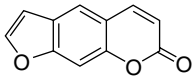
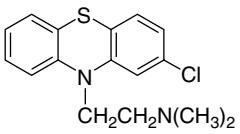
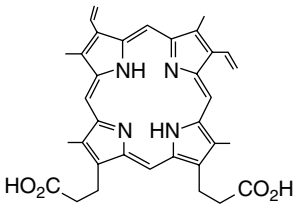
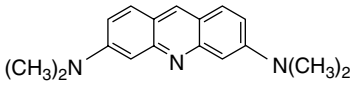
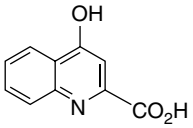
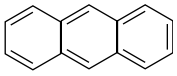
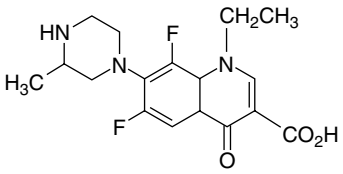
Cyclobutane-type pyrimidine dimers in DNA are the best-studied lesions induced in cells by UV. They are formed predominantly at wavelengths less than 300 nm (Rothman and Setlow, 1979; Rosenstein and Setlow, 1980; Kantor et al., 1980; Yamada and Hieda, 1992), although they have also been found in human skin exposed *in situ* to UV wavelengths of 340–400 nm (Freeman et al., 1987a). These dimers result from the formation of covalent bonds between adjacent pyrimidines of the same DNA strand and interfere with normal DNA function. Beukers and Berends (1960) first demonstrated the formation of these dimers *in vitro*, and Wacker et al. (1960) found them in DNA from UV-irradiated bacteria. These findings marked the beginning of a new era in molecular biology. Pyrimidine dimers were later shown to occur in a number of higher systems, including mammalian (Pathak et al., 1972) and human skin (Freeman et al., 1987b) after UV irradiation.

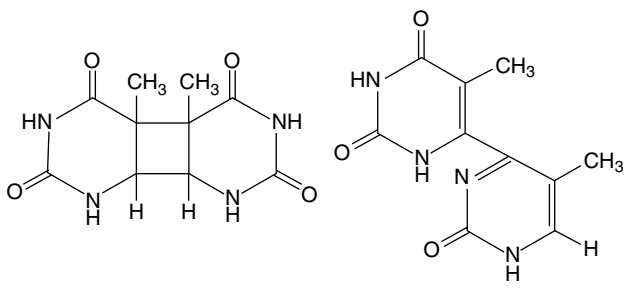
Studies initiated by Cleaver and Trosko (1970) demonstrated the involvement of thymine dimers (TT) (Figure 72.6a) in the disorder xeroderma pigmentosum (XP). This finding represents one of the rare cases in which a specific molecular lesion can be correlated with a malignant process. In another approach, Hart et al. (1977) used cell extracts from UV-irradiated Amazon mollies (small fish) and reported evidence that pyrimidine dimers in DNA gave rise to tumors.

Until recently, sensitive assays for pyrimidine dimers required use of radioisotopes. However, additional techniques have now been developed for measuring pyrimidine dimers. These methods include radioimmunoassays (Mitchell and Clarkson, 1981) and endonuclease digestion followed by determination of DNA chain length (D'Ambrosio et al., 1981; Freeman et al., 1986), which have made quantitation of pyrimidine dimers in human biopsies feasible.

Several possible reaction mechanisms for the sensitized photodimerization of pyrimidines have been suggested, including population of the triplet state of a suitable sensitizer (Lamola, 1968). Our previous work showed that a Schenck type of mechanism (Schenck, 1960) involving a complex-forming reaction is highly favored in photosensitized thymine

TABLE 72.1
Mechanisms and Targets of Selected Groups of Phototoxic Compounds

Compound	Structure	Mechanism of Phototoxicity	Cellular Target	Reference
Psoralen		Direct addition	DNA	Pathak et al. (1974)
		Photodynamic	DNA, membranes, proteins, ribosomes	De Mol et al. (1981) Poppe and Grossweiner (1975), Singh and Vadasz (1978), Pathak (1982)
Phenothiazines		Stable (toxic) photoproduct	DNA	Kochevar (1981)
		Photodynamic	DNA, membrane	Kochevar (1981), Copeland et al. (1976)
Porphyrins		Photodynamic	DNA, membranes proteins	Spikes (1975), Verweij et al. (1981), Jori and Spikes (1981)
Dyes		Photodynamic	DNA, membranes, proteins	Hass and Webb (1981), Ito (1978), Wacker et al. (1964), Wagner et al. (1980),
Kynurenic acid		Photodynamic	Membranes	Wennersten and Brunk (1977, 1978), Pileni and Santus (1978)
Anthracene		Photodynamic	DNA, membranes	Allison et al. (1966), Blackburn and Taussig (1975)
Fluoroquinolones		Photodynamic	DNA, membranes	Rosen et al. (1997), Ouedraogo et al. (1999)



(a) (b)

FIGURE 72.6 Structures of (a) the thymine dimer (cis, syn) and (b) the (6–4) photoproduct.

dimer formation (Kornhauser and Pathak, 1972; Kornhauser et al., 1974). Also, we found that only a few of the potential sensitizers caused measurable thymine dimerization. A small amount (1–2%) of thymine dimer was detected after UV irradiation, even in the absence of a sensitizer. Acetone, ethyl acetoacetate, and dihydroxyacetone were more potent sensitizers than acetophenone and benzophenone (Table 72.2).

The following conclusions can be derived from our results:

1. The sensitized energy transfer taking place during thymine dimerization most likely does not occur through a simple physical mechanism. The ability

TABLE 72.2
Formation of Thymine Dimers (TT) after Irradiation of [2–^{14}C]Thymine in the Presence of Different Sensitizers

Number	Sensitizer	TT Formed (%)
1	None	1–2
2	Acetone	30–40
3	Dihydroxyacetone	25–30
4	Acetophenone	5–10
5	Benzophenone	5–8
6	4-Methoxyacetophenone	2–4
7	Ethyl acetoacetate	35–45
8	Phenyl cyanide	1–3
9	Carbazole	3–6
10	Fluorene	2–3
11	Naphthalene	1–3
12	Xanthene-9-one	1–3
13	Urocanic acid	1–3

Note: Solutions of [2–^{14}C]thymine (2×10^3 M) were irradiated with a total UV ($\leq$ 300 nm) dose of 1.2 J/cm^2. Irradiations were carried out in water (sensitizers 1, 2, 3, and 13), water and ethanol (3:1) (sensitizers 4 and 6–12), and water and diozane (3:1) (sensitizers 5 and 12).

of the sensitizer in its excited state to form a complex with the pyrimidine molecule appears to be a prerequisite for this type of photosensitization.

2. Ethyl acetoacetate and dihydroxyacetone, molecules that are commonly present in any viable cell and were not previously known to be photo sensitizers, proved as effective as acetone or acetophenone. However, urocanic acid, a major UV-absorbing compound in mammalian skin, did not show sensitizing ability in inducing thymine dimerization. The UV energy absorbed by urocanic acid is believed to induce its *cis–trans* isomerization (Baden and Pathak, 1967).
3. Topical preparations containing acetone, dihydroxyacetone, or other acetone derivatives should be used cautiously, since they might damage the epidermal DNA when skin is exposed to UV radiation. Interestingly, one of these compounds, dihydroxyacetone, has been used in cosmetics, notably as the active component in "sunless" tanning lotions (Maibach and Kligman, 1960).

The studies discussed have practical application for correlating the structure of a potential phototoxic agent with its ability to induce pyrimidine dimerization or other molecular lesions in cells.

Other interesting photoproducts of DNA have been isolated and characterized. When a solution of DNA or a frozen thymine solution is irradiated, a new absorption peak at 320 nm appears. This is due to the photochemical formation of new products, the (6–4) adducts. In the case of thymine, 6,4′-(5′-methylpyrimidin-2′-one)-thymine is formed (Figure 72.6b) (Franklin et al., 1982). These compounds cannot be split by reirradiation at short wavelengths as can cyclobutane-type pyrimidine dimers. An additional diagnostic property of these compounds is their instability in hot alkali (Franklin et al., 1982). The (6–4) photoproducts are also generally produced less efficiently than are pyrimidine dimers (Franklin et al., 1982). More recently, there has been an increased interest in the (6–4) adduct-type lesions as they have been shown to play a major role in UV-induced mutagenesis at specific sites in DNA (Franklin and Haseltine, 1986). They used the application of DNA sequencing procedures in *Escherichia coli* to demonstrate that the (6–4) adduct was the mutagenic lesion at certain "hot spots" in the *lacI* gene, a mutation that was previously ascribed to cyclobutane pyrimidine dimers. The relative importance of (6–4) adducts in the lethal and mutagenic effects of UV light, as well as current methods for detection and quantitation, have been discussed in a review (Mitchell and Nairn, 1989).

72.5.2 DNA–Protein Cross-Links

The previous discussion focused on the reaction between bases, specifically thymine, within a strand of DNA to form an adduct. However, DNA in the cell has a complex and varied environment, making possible additional light-induced reactions.

Heteroadducts of DNA are those adducts formed by the covalent attachment of different types of compounds to DNA. These adducts may involve cellular constituents such as proteins, or exogenous compounds such as drugs, food additives, and cosmetics. Heteroadducts may have profound effects on cells. Artificially produced covalent linkages like DNA–protein cross-links, of the type not observed in normal viable cells, may result in a phototoxic response or be expressed as mutagenic or carcinogenic events.

The chemical nature of the DNA–protein cross-links is not yet known. An *in vitro* photochemical reaction between thymine and cysteine has been observed (Schott and Shetlar, 1974) and may be one of the mechanisms for covalent linking of DNA to protein *in vivo* (Smith, 1974). Similarly, it has been reported that irradiation of thymine-labeled DNA and lysine in aqueous solvent produces a photoproduct that behaves like a thymine–lysine adduct (Shetlar et al., 1975). Furthermore, 11 of the common amino acids combine photochemically with uracil in different model systems (Smith, 1974). These pyrimidine-amino acid adducts are regarded as models for the coupling sites between proteins and DNA. In addition to reactions directly induced by UV, model systems provide evidence that acetone and acetophenone are effective photosensitizers for the covalent addition of amino acids to pyrimidine bases (Fisher et al., 1974). It is reasonable to assume that suitable chromophores present in drugs, cosmetics, etc., will also be able to photosensitize the cross-linking of proteins and nucleic acids *in vitro* and *in vivo*.

The cross-linking of DNA and protein in bacteria was the first *in vivo* photochemical heteroadduct reaction reported (Smith, 1962). Several studies of UV-induced DNA and

protein cross-links in mammalian cells *in vitro* have been based mainly on reduced DNA extractability after UV irradiation (Todd and Han, 1976). Evidence that this lesion plays a significant role in killing UV-irradiated cells has been obtained under several experimental conditions.

Mammalian (eukaryotic) cells, in general, represent a suitable model for the cross-linking reaction. Within the nuclei of eukaryotic cells, DNA is in intimate contact with proteins responsible for structurally organizing DNA and controlling macromolecular synthesis. Such a DNA–protein complex is commonly referred to as chromatin. The proximity of nuclear proteins to DNA should facilitate the formation of UV-induced DNA–protein covalent bonds. Todd and Han (1976) studied the general features of UV-induced (254 nm) DNA–protein cross-links in asynchronous and synchronous HeLa cells. Cross-linking was demonstrated by the detection of unextractable DNA in irradiated cells. Fornace and Kohn (1976), using a sensitive alkaline elution assay, measured UV-induced DNA–protein cross-links in both normal and xeroderma pigmentosum human fibroblasts. They noted that normal cells exhibit a repair phase lacking in XP cells. Similarly, Peak and Peak (1989) have reported DNA–protein cross-linking in cells exposed to UVA, UVB, or UVC radiation. These workers reported the relative importance of several DNA lesions (thymine dimers, single-strand breaks, and DNA–protein cross-links) for each spectral region. DNA–protein cross-links were found to be the lesion most efficiently produced by UVA-irradiation of cells.

No *in vivo* data on DNA–protein cross-linking in mammalian skin, other than our preliminary work, have been reported. To study the possible role of the DNA–protein cross-links in epidermis, we focused on the isolation of chromatin from irradiated and nonirradiated guinea pig skin (Kornhauser, 1976; Kornhauser et al., 1976a). The epilated backs of guinea pigs were irradiated with a moderate physiological dose (80 mJ/cm^2; 290–350 nm) that corresponds to approximately four times the minimal erythema dose in an average fair-skinned Caucasian. Epidermis was obtained from both the irradiated and the control (nonirradiated) sites on the same animal and was homogenized. Chromatin was isolated from the homogenates by using Sepharose B-4 and DEAE cellulose chromatography and density gradient centrifugation. Its biological activity was determined by chemical and biochemical methods (Kornhauser et al., 1976a). We were able to obtain 4–5 mg of extractable DNA, which was free of protein, from 1 g wet epidermal tissue. Immediately after UV irradiation, the yield of extractable DNA was reduced by 20–30%, presumably as a result of DNA–protein cross-linking and possibly of DNA strand breakage. The latter molecular lesion is consistent with previous findings (Zierenberg et al., 1971). In this experiment we found (1) a significant breakdown of the high-molecular-weight DNA fraction and the presence of low-molecular-weight DNA fragments on top of the sucrose gradient after UV-irradiation, and (2) an increment in the high-molecular-weight DNA isolated 60 min after irradiation (the regeneration or repair phase).

The results discussed can be summarized as follows:

1. UV irradiation, at physiological doses (4 MED) of 290–350 nm, decreased the actual amount of dissociable chromosomal DNA by 20–30% as a result of DNA strand breakage and cross-linking of DNA to protein.
2. A comparison of corresponding elution profiles from Sepharose columns of dissociable DNA isolated from UV-irradiated and nonirradiated epidermal specimens indicated cross-linking of protein to DNA.
3. UV irradiation caused significant breakdown of the high-molecular-weight DNA that was isolated after irradiation.
4. In the regeneration phase, an active repair of strand breaks and possibly DNA–protein heteroadducts was operating in the viable cells of the epidermis.

So far, no other evidence for the cellular repair of DNA–protein heteroadducts has been found *in vivo.* It is conceivable that cells exposed to light have evolved a repair system for eliminating this type of heteroadduct. It is likely that this system is different from photoreactivation, which is specific for pyrimidine dimers (Setlow and Setlow, 1963).

All these findings suggest that UV radiation, even in moderate doses, can induce measurable alterations of the chromosomal material chromatin in mammalian skin. At present, it is not known what biochemical changes accompany light-induced lesions in chromatin. It is possible that damage by photons may alter such important chromatin functions as regulation of gene expression. Thus further studies of lesions in chromatin are indispensable for a complete understanding of light-induced effects on cells.

72.5.3 Phototoxicity

In addition to DNA–protein cross-linking, cross-links between DNA strands are possible. Because of the distance between bases in the DNA double helix, light-induced cross-linking is not observed without a bridging molecule such as a drug or component of a cosmetic, etc. Psoralens, a class of furocoumarins, are important cross-linking agents. Psoralens are a group of naturally occurring and synthetic substances that, when added to biological systems and irradiated with UVA, produce various biological effects. These effects are not observed with either psoralens or light alone.

The photobiological reactions of psoralens with DNA have received widespread attention in recent years. On the molecular level, the following facts are known:

1. Psoralens intercalate into DNA, that is, slip in between adjacent base pairs by forming molecular complexes involving weak chemical interactions ("dark reaction").
2. UVA irradiation of the DNA–psoralen complex, *in vivo* or *in vitro,* results in covalent bond formation

between a pyrimidine base and the furocoumarin molecule (C_4 cycloaddition). Because of their structure, psoralens in this reaction can react either at their 3,4 double bond or at their corresponding 4-~,5-~ site, yielding monoadducts (in the former case the product is not fluorescent, and in the latter case it is).

3. The absorption of an additional photon may result in a further chemical reaction yielding a "cross-linked DNA." Thus psoralens can behave as photoreactive bifunctional agents; one psoralen molecule reacting with two pyrimidines in opposite strands of DNA. The structures of psoralen mono- and di-adducts with thymine are shown in Figure 72.7. Figure 72.8 schematically shows DNA cross-linked by a psoralen molecule. The result is a cross-linked DNA in which the individual strands cannot be separated by standard denaturation conditions. Both types of lesions, the monofunctional adduct and the cross-linked product, can be repaired *in vivo* (Pathak and Kramer, 1969; Baden et al., 1972) and *in vitro* (Friedburg, 1988).

Dall'Acqua (1977) showed that the photoaddition of furocoumarins to DNA is not a random process. Specific sites exist in DNA for the photochemical interaction with psoralens. The sites that can be considered specific receptors for the photobiological activity of psoralens are represented by alternating sequences of adenine and thymine in each complementary strand of the polynucleotide. Psoralen has a greater photoreactivity toward thymine than it has toward cytosine. The receptor sites have a high capacity for intercalation and subsequent photoreaction with psoralens (Dall'Acqua, 1977). It has been shown that flanking sequences, in addition to the adenine and thymine content of DNA, determine cross-linking (Boyer et al., 1988).

The covalent addition of psoralens to DNA, particularly the cross-linking reaction, is usually believed to be responsible for the major effects of psoralen photosensitization. These include mutation and lethality in prokaryotic and eukaryotic systems, inhibition of DNA synthesis, sister chromatid exchange, and carcinogenesis. However, the relationship between psoralen photoaddition to DNA and the appearance of erythema remains to be elucidated. From early studies it appeared that erythema, the basic phototoxic effect induced by psoralens, correlated well with the *in vitro* capacity of psoralen derivatives to bind covalently to DNA (Vedaldi et al., 1983). Neither the specific photoproduct(s) required for initiating psoralen-induced erythema nor the subsequent molecular events (e.g., mediators involved) have been definitely established.

Initially, the ability to sensitize cutaneous tissue appeared to be a unique characteristic of the psoralen ring system; for instance, pyranocoumarins, which have a similar linear tricyclic ring system, are found to lack photosensitizing activity (Pathak et al., 1967). Furthermore, cutaneous phototoxicity is usually expressed only with linear derivatives; the angular furocoumarin, angelicin, does not photosensitize mammalian

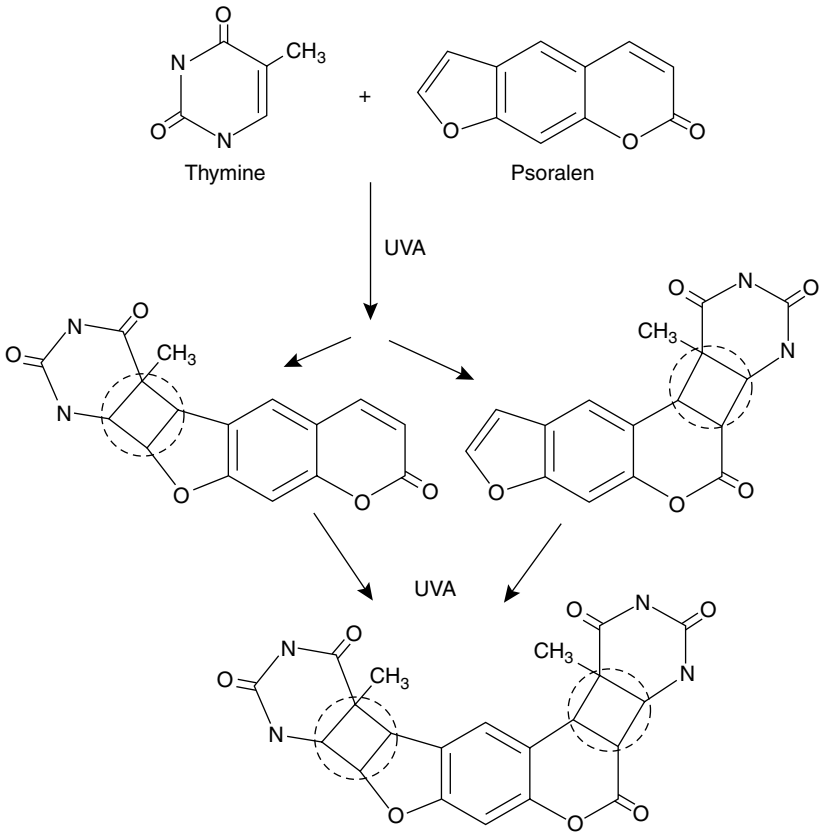


FIGURE 72.7 Photoaddition products of psoralen with thymine after UV irradiation.

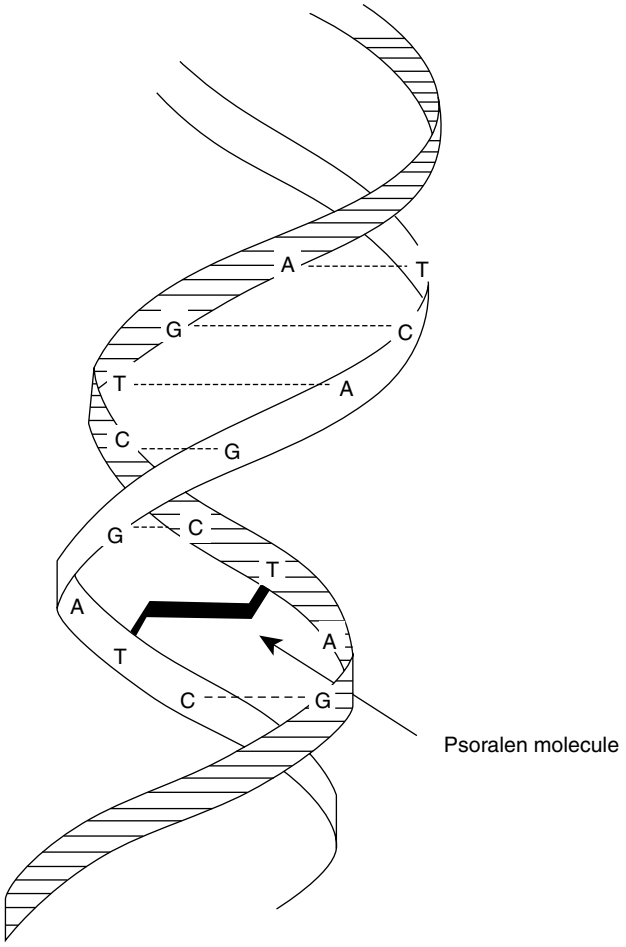


FIGURE 72.8 Schematic representation of DNA cross-linked by a psoralen molecule.

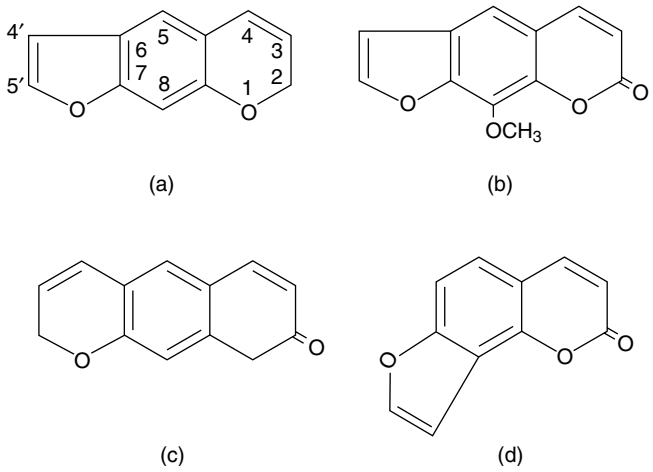


FIGURE 72.9 Structures of some furocoumarins and a pyranocoumarin: (a) psoralen; (b) 8-methoxypsoralen (8-MOP); (c) pyranocoumarin; (d) angelicin.

skin (Dall'Acqua et al., 1981). Small changes in the structure of psoralen may produce dramatic changes in photosensitizing ability. Unsubstituted psoralen causes the most severe phototoxicity. This photobiological activity is reduced by adding methyl (on carbon 3) or halogen substituents (Pathak et al., 1967). The structures of some furocoumarins and pyranocoumarin are shown in Figure 72.9.

The correlation of the structure of psoralen photoproducts to their photobiological effects has been the topic of several investigations. A large number of synthetic and natural furocoumarins have been subjected to systematic studies. From this work it has been concluded that the erythemogenic effect correlates with the capacity of a furocoumarin to form cross-links rather than monoadducts to DNA (Vedaldi et al., 1983). This fact was confirmed by several investigators by preparing a relatively large number of monofunctional furocoumarin derivatives and testing their photobiological properties (Rodighiero et al., 1984).

In general, the monofunctional compounds do not induce erythema in human and guinea pig skin. Although this fact has been experimentally verified in many cases, exceptions to this rule seem to exist. A few 4-methyl angelicin derivatives are able to strongly photoreact with DNA without forming cross-links. When tested on guinea pig skin they were able, under certain experimental conditions, to induce a mild erythema (Baccichetti et al., 1981, 1984). We must point out, however, that those experiments involved topical application of the compound in a relatively high concentration and with a high UVA dose. Also, great care must be taken in these experiments to ensure that the sample is free from bifunctional psoralen impurities, as they can easily yield false-positives. In summary, a simple concept such as cross-links = erythema, monoadduct = no erythema has yet to be established.

Various derivatives of psoralen, some with photosensitizing activity, have been synthesized. The synthesis of these derivatives is largely driven by the need to find new agents for improving current photochemotherapeutic treatment regimens. These derivatives include benzopsoralens and their tetrahydro-derivatives, pyrrolocoumararins, azapsoralens, thiopsoralens and khellin, and related methylfurochromones (Dall'Acqua, 1989; Vedaldi et al., 1997). The photobiological activity of many of these novel compounds has to be investigated. Several derivatives (such as 1-thiopsoralen, 4-hydroxymethyl-4'-methylpsoralen, azapsoralens, and benzopsoralens) exhibit the ability to inhibit cellular growth, while eliciting no, or mild, erythemal responses (Conconi et al., 1996; Bordin et al., 1992; Chilin et al., 1999; Dalla Via et al., 1999). These compounds could potentially provide effective photochemotherapeutic treatment without adverse effects, such as erythema. The driving force behind these investigations is to find new agents with the potential for improving current photochemotherapeutic treatment regimens.

Alternative mechanisms for the induction of erythema by psoralens and UV light that do not involve photoaddition to DNA have been suggested. One alternative mechanism is derived from the observation that there is a relationship between erythema production and the ability of a compound to form $^1O_2$ (Pathak, 1982). This correlation suggests that $^1O_2$ may be a mediator in psoralen-induced erythema. The involvement of $^1O_2$ in psoralen phototoxicity, however,

has yet to be conclusively proven. Indeed, it has been pointed out that both the production of $^1O_2$ and the monoadduct and, particularly, the diadduct formation proceed by way of a common intermediate, the psoralen triplet state (De Mol et al., 1981). Thus, psoralens that undergo efficient intersystem crossing should readily photosensitize the formation of $^1O_2$, as well as photoreact with DNA, unless low DNA binding or steric constraints predominate. It is, therefore, understandable that reports of correlation between both $^1O_2$ formation and erythema production (Pathak and Joshi, 1984), as well as DNA photobinding and erythema production (Vedaldi et al., 1983), have appeared. However, the causal relationship between these two photoproducts ($^1O_2$ or DNA adducts) and erythema production is still under active investigation. Another alternative mechanism, not involving direct addition to DNA, has been proposed (Laskin et al., 1986). It was demonstrated *in vitro* that 8-MOP binds to a specific cell surface receptor, thus inhibiting epidermal growth-factor binding. This work demonstrated for the first time that 8-MOP in combination with UVA irradiation can modify cell surface receptors in a variety of human and mouse cell lines. This alteration may play an important role in the mechanism of psoralen phototoxicity.

Two studies have been completed in our laboratory that have focused on psoralen + UVA (PUVA)–induced phototoxicity. Both involved the micro-nutrient β-carotene, a naturally occurring pigment and vitamin A precursor found in many green and yellow-orange fruits and vegetables. Beta-carotene is a well-established quencher of $^1O_2$ and photooxidation (Krinsky and Deneke, 1982). In the first experiment, we studied the potential of β-carotene to influence PUVA-induced erythema in rats (Giles et al., 1985). The rats were fed an β-carotene-fortified diet for approximately 14 weeks before treatment. Levels of β-carotene accumulated in the skin were measured by high-performance liquid chromatography (HPLC). The rats were then orally dosed with β-MOP (20 mg/kg body weight, in corn oil) and were irradiated 2 h later with a single dose of UVA (5 J/cm^2). We found that the animals on the β-carotene fortified diet were significantly protected against PUVA-induced erythema. Furthermore, those rats having the highest β-carotene skin levels showed no perceptible erythema, indicating a correlation between β-carotene skin levels and a protective effect. No such protective effect was observed against UVB-induced erythema.

In the second experiment, we investigated the potential of β-carotene for decreasing PUVA-induced melanogenesis (Kornhauser et al., 1989). One of the side effects of PUVA therapy is tanning of the skin caused by an increase in the number of epidermal melanocytes (Blog and Szabo, 1979). Melanin is formed by an oxidative process. Previous *in vitro* studies indicated a role of activated-oxygen species in melanin synthesis (Kornhauser et al., 1976b). Therefore, we reasoned that 8-carotene might be able to influence melanogenesis *in vivo*. The animal of choice was the C57 BL/6 mouse, since the tail-skin had been found to be a good model for melanogenesis in human skin (Szabo et al., 1982). Mice were fed standard rodent chow diets supplemented with either 1% β-carotene beadlets or 1% placebo beadlets for 10 weeks before treatment and throughout the treatment period. Mice were divided into UVA- and PUVA-treated groups. PUVA-treated mice received 20 mg/kg body weight of 8-MOP in corn oil orally by intubation, followed 2 h later with 3 J/cm^2 of UVA irradiation of the tail. The body of the animal was shielded from the light. UVA-treated mice received 3 J/cm^2 of UVA only. Mice received 2, 4, or 5 treatments within a 3-week period. Selected mice from each group were euthanized after these treatments and the tail-skin epidermis was removed for dihydroxyphenylalanine (DOPA) histochemical processing (Staricco and Pinkus, 1957). Melanogenesis was evaluated by counting the number of DOPA-positive melanocytes. As expected, the PUVA treatment resulted in an increase in the number of DOPA-positive melanocytes counted in each tail-skin epidermal section. An increase was also observed in the UVA-treated mice. However, mice fed β-carotene in both the UVA- and PUVA-treated groups had significantly fewer ($p < .05$; Student's *t*-test) DOPA-positive melanocytes than the corresponding placebo-fed animals at all three time points. The results are presented in Figure 72.10.

The most direct interpretation of the results described in both of these experiments would be that PUVA treatment involves photooxidation via $^1O_2$ or free radicals. It is well known that β-carotene is an effective quencher of these reactive intermediates (Krinsky and Deneke, 1982; Burton and Ingold, 1984). However, there is an alternative explanation for the observed protective effect, which involves the quenching of the psoralen triplet state by â-carotene (Giles et al., 1985). Further studies are in progress to ascertain the mechanism for the β-carotene protective effect and the role of $^1O_2$ in PUVA-induced phototoxicity. In summary, the role of $^1O_2$ and related species in the induction of PUVA-induced erythema and other photobiological effects needs to be more extensively investigated before definitive conclusions can be drawn.

Some interesting studies on the mechanism of PUVA-induced melanogenesis involving photoreactions between furocoumarins and membrane unsaturated fatty acids (UFAs) have been performed (Dall'Acqua and Martelli, 1991). The C4-cycloaddition between one of the olefinic bonds of UFAs and the pyrone-side double bond of psoralen takes place after UV irradiation at a wavelength of 365 nm. This photoreaction also occurs between psoralen and the UFAs in lecithin *in vitro* and is indicated by indirect evidence *in vivo*. Caffieri et al. (1994) suggested that these lesions of cell membrane components might play a role as a second messenger, mimicking diacylglycerol (DAG). Recognizing the structural similarity between DAG and the psoralen-UFA cycloadducts, these investigators compared DAG's effective activation of protein kinase C (PKC) to that of the cycloadducts for activation of PKC in human platelets. Treatment of intact platelets with DAG resulted in the activation of PKC as measured by phosphorylation of a 47 kDa protein, which is the major substrate for PKC. Results

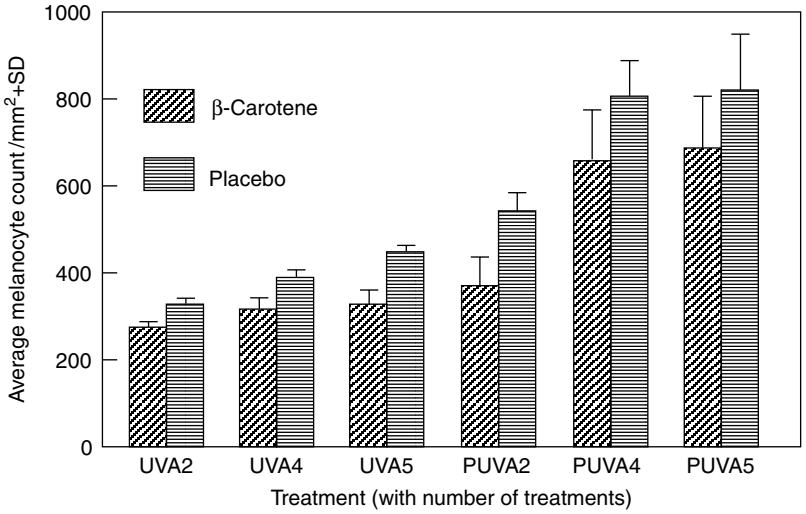


FIGURE 72.10 Average melanocyte counts as a measure of the effects of diet and treatment on DOPA-processed tail-skin epidermis of C57BL/6 mice. Averages are shown with the corresponding standard deviation. Mice were fed either β-carotene or placebo-fortified diets. Treatment groups: UVA-light treated (UVA), and oral 8-methoxypsoralen + UVA-light treated (PUVA). Mice received two, four, or five treatments. The numbers of DOPA-positive melanocytes were counted using a light microscope.

from the same platelet system showed that phosphorylation was induced to a similar extent by substituting DAG with the psoralen-linoleic acid cycloadduct. Gordon and Gilchrest (1989) reported previously that DAG stimulated melanogenesis in cultured melanocytes. In summary, these studies suggest that psoralen UFA photoadducts may affect melanogenesis and that this mechanism may play an important role in PUVA-induced tanning.

Skin photosensitization is one of the most widely studied properties of furocoumarins. Several types of photodermatoses occur when skin comes into contact with plant or vegetable products containing psoralens and is later exposed to sunlight. Much less is known about potential adverse cutaneous effects resulting from chronic ingestion of foods that contain furocoumarins, such as figs, limes, parsnips, and cloves.

Although furocoumarins are potent phototoxic compounds, they are also used as therapeutic agents. Because of their ability to induce melanogenesis, psoralen derivatives have been applied clinically to treat vitiligo (leukoderma) and increase the tolerance of human skin to solar radiation. A new clinical discipline, photochemotherapy (PCT), is increasingly being introduced to treat psoriasis and other skin disorders (Parrish et al., 1974; Wolff et al., 1976; Gilchrest et al., 1976).

Photochemotherapy involves the controlled interaction of light and orally administered drugs to produce beneficial effects. Psoralen PCT has entered the medical terminology as PUVA. The PUVA regimen is effective, clean, and acceptable to patients. However, some problems persist; these include possible induction of cataracts (Cloud et al., 1960; Stern, 1994), hematologic effects (Friedmann and Rogers, 1980), alteration of the immune response (Strauss et al., 1980; Aubin and Humbert, 1998), and skin aging (Bergfeld, 1977). In addition, epidemiologic studies have demonstrated that extended treatment with PUVA can increase a patient's risk of basal cell carcinoma and malignant melanoma (Stern et al., 1979, 2001; Stern, 1989; Hönigsmann et al., 1980).

The use of psoralens in PCT has raised some additional questions concerning their phototoxicity. The structurally similar psoralens, 8-MOP, 5-methoxypsoralen (5-MOP), and 4,5′,8-trimethylpsoralen (TMP) have similar topical phototoxicity. However, when they are orally administered, the phototoxicity of TMP and 5-MOP is greatly diminished compared to that of 8-MOP (Mandula et al., 1976; Hönigsmann et al., 1979). This has been exploited by two European teams, who introduced 5-MOP as an alternative to 8-MOP, in the PCT of psoriasis (Hönigsmann et al., 1979; Grupper and Berretti, 1981). Although the clearing of psoriatic lesions was comparable with 5-MOP and 8-MOP, acute side effects (including phototoxicity) were significantly reduced in the 5-MOP regimen. As more has been learned about the biotransformations of psoralens (Mandula et al., 1976), it appears that metabolism may play a central role in determining the relative oral phototoxicity of substituted psoralens. However, it has not been established that reduced delivery of the phototoxic psoralen to the epidermis, due to metabolism or lack of absorption, is the basis for the observed differences in oral phototoxicity.

We have reported serum and epidermal levels of 5-MOP and 8-MOP in guinea pigs (Kornhauser et al., 1982). Determinations of psoralen levels in the epidermis, the primary target organ for phototoxicity, had not previously been reported for either humans or an animal model. For this study, we chose a guinea pig model system that we and others (Harber, 1969) have found to be reliable for predicting phototoxicity in humans. Our results indicated that, after equivalent oral dosing, metabolism or absorption constrains

5-MOP to lower epidermal levels than 8-MOP. Therefore, by orally administering 5-MOP it should be possible to maintain epidermal drug concentrations at lower levels than in an 8-MOP regimen.

Because psoralens, as used in PCT, react covalently with DNA, there is a potential risk of mutagenicity and oncogenicity. Indeed, in an *in vitro* study, 8-MOP and 5-MOP exhibited essentially the same activity in inducing chromosome damage in human cells (Natarajan et al., 1981). Furthermore, it was reported that topical 5-MOP combined with UVA-induced carcinogenesis in mice comparable to that observed with 8-MOP (Zajdela and Bisagni, 1981). These two studies suggest that 5-MOP and 8-MOP have a similar oncogenic potential when topically administered.

Extrapolating our findings with orally dosed guinea pigs to clinical applications, we suggest that a 5-MOP therapeutic regimen may minimize damage to epidermal DNA, reducing the risk of carcinogenesis that is suspected in 8-MOP PCT. For this reason, and because of the reduced acute side effects in a 5-MOP regimen, we feel that 5-MOP should be tested further, along with other psoralen derivatives, as alternatives to 8-MOP in PCT.

An additional application of psoralen phototoxicity, extracorporeal photophoresis, is increasingly being used for management of disorders such as cutaneous T-cell lymphoma (Edelson, 1988). Photophoresis involves oral administration of 8-MOP, then withdrawal of 1 unit of blood 2 h later. The blood is separated into its components by centrifugation. Plasma and leukocytes are combined with saline. This suspension is then passed as a thin film between twin banks of high-intensity UVA lamps. After irradiation, the erythrocytes are recombined with the remainder of the blood and retransfused into the patient. It has been reported that photophoresis is an effective treatment in many instances (Edelson et al., 1987; Oliven and Shechter, 2001). The mechanism of this therapy appears to be complex, not merely involving cytotoxicity but also immunologic effects.

72.5.4 Photosensitized Oxidations

Many phototoxic compounds, such as porphyrins and dyes, affect biological substrates through photosensitized oxidations. These substances absorb light (both in long-wavelength UV and visible regions) and sensitize photooxidization from their triplet excited states. Following excitation, there are two distinct mechanisms (types I and II) that result in photooxidation (Figure 72.11).

Although opinion is divided, type II is probably the more common mechanism producing $^1O_2$, a highly reactive oxidizing agent. A unique feature of $^1O_2$ involvement in photodynamic action is the fact that the generation and reaction sites may be different, the diffusion range of $^1O_2$ in cytoplasm being in the order of 0.1:m (Moan et al., 1979). In contrast, in type I (radical) mechanism, the sensitizer and substrate must be closer at the time of photon absorption. The major processes involving $^1O_2$ are photooxidative loss of histidine,

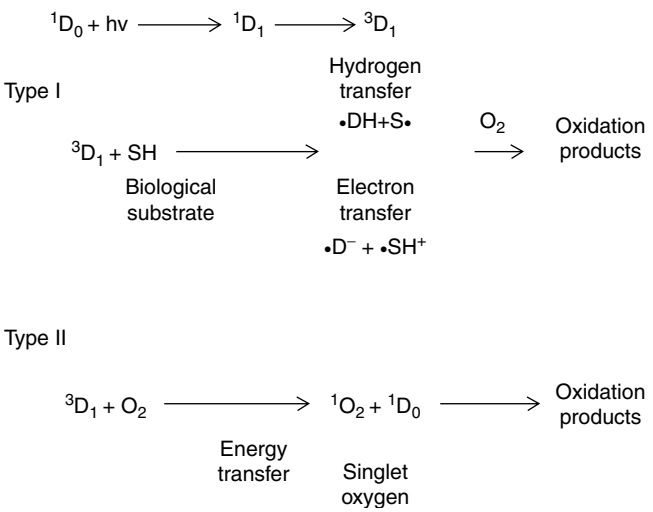


FIGURE 72.11 Mechanisms of photosensitized oxidation. The ground state sensitizer ($^1D_0$) is excited to the lowest excited singlet state ($^1D_1$) and undergoes intersystem crossing to the lowest excited triplet state ($^3D_1$).

methionine, tryptophan, tyrosine, and cysteine in proteins; photooxidation of guanine bases in DNA; and formation of hydroperoxides with unsaturated lipids.

It has been recognized for decades that membrane damage plays a role in the photoinactivation of cells, especially in the presence of photodynamic sensitizers (Raab, 1900; Blum, 1941). The mechanism of cell membrane damage and disruption has been extensively studied for several photodynamic sensitizers. Photohemolysis of red blood cells sensitized by protoporphyrin (metal-free porphyrin) has been studied extensively, because in several inheritable diseases of porphyrin metabolism (porphyrias), the red cells contain unusually high levels of photosensitizing porphyrins. Oxygen is required for protoporphyrin-photosensitized red cell lysis. On the molecular level, it is known that $^1O_2$, formed by energy transfer from triplet state protoporphyrin in red blood cell membranes, oxidizes unsaturated lipids (Lamola et al., 1973; Goldstein and Harber, 1972). Incorporation of cholesterol hydroperoxides, such as those formed in cholesterol photooxidation by protoporphrin, leads to increased osmotic fragility and hemolysis of red blood cells (Lamola et al., 1973). Protoporphyrin has also been shown to photosensitize protein cross-linking in membranes (Verweij et al., 1981). It has been suggested that additional, more subtle, membrane functions, such as active transport of small molecules, are altered by membrane protein cross-linking (Kessel, 1977; Lamola and Doleiden, 1980). Indeed, later *in vitro* studies have shown that such photodynamic sensitizers can inactivate single ion channels and, also, transport sugars and amino acids across cellular membranes (Kunz and Stark, 1997; Specht and Rodgers, 1991; Paardekooper et al., 1993).

Photooxidation of cell membrane components and proteins is not the only mode of photodynamic damage. Various photodynamic sensitizers were found to be mutagenic

in bacteria (Gutter et al., 1977), yeast (Kobayashi and Ito, 1976), and mammalian cells (Gruener and Lockwood, 1979; Paardekooper et al., 1997; Takeuchi et al., 1997; Jeffrey et al., 2000). Thus direct photodynamic damage to DNA is suspected, although alternative mechanisms for photodynamic mutagenesis have been proposed (Mukai and Goldstein, 1976).

There is now abundant evidence that bases in DNA may be oxidized by photodynamic sensitizers in the presence of light. Wacker et al. (1964) presented the earliest direct evidence of photooxidative damage to guanine in DNA. Subsequent studies have demonstrated that, to differing degree, all nucleobases in DNA are susceptible to damage by photochemically generated radicals (i.e., type I photooxidation) (Cadet et al., 1997). However, because guanine is the nucleobase with the lowest ionization potential, oxidative damage to guanine frequently predominates. Photosensitization through a type-II mechanism, in which singlet oxygen is formed, has been shown to result in oxidative damage only to guanine bases in DNA (Cadet et al., 1997). Because photosensitized oxidation through both type I and II mechanisms results in damage to guanine bases, photoproducts of guanine have been widely used as markers for photooxidative damage in DNA. A number of products of the photooxidation of guanine have been isolated and characterized (Cadet and Teoule, 1978). The predominate decomposition product of guanine oxidation in DNA has been found to be 8-oxo-7,8-dihydro-2~-deoxyguanosine (8-oxodG) (Cadet et al., 1997). With the development of sensitive and rapid methods for quantifying its formation (Floyd et al., 1986), 8-oxodG has become the most widely used biomarker for oxidative damage to DNA. To date, this approach has been used to investigate a variety of photosensitizers including methylene blue (Floyd et al., 1989), hematophorphyrin D (Floyd et al., 1990), riboflavin (Yamamoto et al., 1992), rose bengal (Schneider et al., 1993), fluoroquinolones (Rosen et al., 1997), furocoumarins (wamer et al., 1995) and titanium dioxide (Wamer et al., 1997). These studies promise to better define the role of photooxidative damage to DNA in phototoxicity and photomutagenesis.

The detailed mechanism of photooxidation of bases in DNA is not fully understood. When cells are in an environment containing a photodynamically active chromophore, such as a porphyrin or toluidine blue and exposed to visible light, damage to DNA from $^1O_2$ might be expected to result from an extracellular as well as an intracellular sensitizer. However, it has been found that toluidine blue, which is not taken up by cells, does not damage DNA (Ito and Kobayashi, 1977). Porphyrins, however, accumulate in cells and the efficiency of inducing DNA lesions follows the cellular uptake curve (Moan and Christensen, 1981). It is generally felt that accessibility of the sensitizing dye to DNA is a major factor in determining photomutagenic potential.

Both types I and II mechanisms have been proposed for the photooxidation of DNA. The major pathway will be determined by the structure of the photosensitizing compound, the extent and type of binding to DNA, the oxygen concentration, and the polarity of the cellular environment (Kochevar, 1981; Ito, 1978).

Recent research has clearly shown that UV radiation can induce oxidative damage in skin without exposure to exogenous photodynamic sensitizers. It has been shown that:

1. UV induces the formation of free radicals in skin. Electron spin resonance spectrometry has been used to demonstrate that superoxide radical anion or hydroxyl radicals are generated in UV-irradiated whole skin (Pathak and Stratton, 1968) and skin homogenates (Ogura and Sugiyama, 1993).
2. The concentrations and oxidation state of antioxidants and antioxidant enzymes are altered after UV irradiation. Shindo et al. (1994) have reported that cutaneous antioxidants (glutathione, tocopherol, and ubiquinone) and antioxidant enzymes (particularly superoxide dismutase and catalase) are partially depleted in the skin of UV-irradiated mice. Depletion of these antioxidant defenses is measurable immediately after irradiation and is therefore not associated with the inflammatory response.
3. The level of oxidatively damaged molecules is elevated in UV-irradiated skin. Lipid peroxidation is significantly elevated after irradiation of mouse (Shindo et al., 1994) or human (Punnonen et al., 1991) skin *in vivo*. In addition, oxidative damage to DNA, measured as the formation of 8-hydoxy-2′-deoxyguanosine, is detected in the skin of UV-irradiated mice (Hattori-Nakakuki et al., 1994).

These observations strongly indicate that UV radiation induces a state of oxidative stress in the skin. The identity of the endogenous photosensitizer(s) in the skin, whose excitation leads to photooxidation, is presently unknown. In addition, the relative importance of photooxidative damage caused by UV irradiation of skin and other molecular lesions (such as thymine dimers and DNA-protein cross-links) is at present unclear. Evidence is emerging that photooxidative stress in the skin may play a significant role in chronic disorders, such as photocarcinogenesis (Black and Mathews-Roth, 1991) and photoaging (Bryce, 1993). However, the causal connection between photooxidation in the skin and significant adverse effects remains to be proven.

Selective photosensitized oxidative damage to cells has effectively been employed in photodynamic therapy (PDT) of solid tumors including eye, bladder, skin, and endobronchial tumors (Dougherty, 1987). PDT involves the use of hematoporphyrin (HP) derivatives as the photosensitizer. The HP derivatives, when injected, localize in tumors. Tissue is then irradiated with intense visible light, usually obtained by using a dye laser conjoined with fiber optics. The therapy described, which involves light activation of therapeutic agents, has the clear advantage of selectivity, that is, only the irradiated tissue is affected.

72.5.5 Mutations and Changes in Cellular Phenotype

The described classes of light-induced damage (thymine dimers [6–4] adducts, DNA–protein cross-links, psoralen-DNA adducts, and oxidation of bases) represent potential premutational sites in DNA. High-fidelity repair of these DNA lesions would eliminate adverse cellular effects. As discussed, repair mechanisms have been found for many light-induced changes in DNA. Alternatively, unrepaired (or incorrectly repaired) DNA damage may lead to a range of cellular outcomes, including no effect (if the genetic alteration is unexpressed), cell death, or transformation to a neoplastic phenotype. The complex sequence of molecular events that determine these cellular outcomes is now becoming understood through the techniques of molecular biology.

Errors in DNA repair, or replication of a damaged DNA template result in the fixation of a DNA mutation. Several types of light-induced DNA mutations have been reported. Point mutations, involving single nucleotide base pair replacements, have been characterized in bacterial systems (Hutchinson and Wood, 1988; Cebula and Koch, 1990), well-defined plasmid sequences (Drobetsky et al., 1989), and in mammalian genes (Bohr and Okumoto, 1988). Frameshift mutations, resulting from the addition or deletion of one or more base pairs, have also been studied (Cebula et al., 1989). Techniques used to define mutational spectra (i.e., types of mutation and specific location within a DNA sequence) include hybridization with highly specific probes (Cebula and Koch, 1990; Pierceall et al., 1991), direct DNA sequencing (Hutchinson and Wood, 1988; Cebula et al., 1989), and analysis of altered restriction endonuclease sites (Drobetsky et al., 1989). Use of polymerase chain reaction (PCR), to amplify DNA sequences within genes of interest, has allowed rapid and sensitive detection of UV-induced mutations in mammalian genes (Brash et al., 1991; Tornaletti and Pfeifer, 1994).

The derived mutation spectra have proven useful for tracing the etiology of skin cancer and understanding mechanistic steps in UV-induced mutations. Brash et al. (1991) have described a point mutation in DNA isolated from human squamous cell carcinomas, which may be the characteristic of UV-induced carcinogenesis. Using PCR to amplify selected exons in the *p53* tumor suppressor gene, followed by direct sequencing of amplified exons, they demonstrated that the characteristic mutations were CC to TT double-base substitutions. This observation of distinctive mutations produced by UV radiation has been subsequently confirmed by several investigators (Kress et al., 1992; Dumaz et al., 1994). This molecular epidemiological approach has significantly increased our understanding of the etiology of human skin cancers. The use of molecular biological techniques has also led to a clear understanding of factors that predispose genes to mutation by UV radiation. As a corollary to the observation that CC6TT mutations occur after irradiation with UV, it has been observed that pyrimidine-rich DNA sequences have increased sensitivity to mutations induced by UV radiation (Brash et al., 1991). In addition, it has been shown that UV-induced mutations in DNA derived from human skin carcinomas predominate the nontranscribed strand of DNA (Dumaz et al., 1993). Tornaletti and Pfeifer (1994) have definitively shown that rates of DNA repair are highly variable within a mammalian gene. Furthermore, they demonstrated that slowly repaired regions of DNA, such as nontranscribed DNA strands, are hot spots for UV-induced mutations. These insights into the factors predisposing UV-damaged DNA to mutation are essential for understanding the mechanism(s) of UV-induced mutations.

In the past decade, our understanding of the genetic basis of cancer has dramatically increased largely because of the discovery of specific genes, protooncogenes, whose normal function is vital for appropriate regulation of cellular growth and differentiation. Alteration of protooncogene structures or the regulation of their expression may lead to cancer (Bishop, 1983).

It is now well established that DNA damage, such as point mutations, can activate oncogenes. The role of oncogenes in UV-induced carcinogenesis is currently under active investigation. UV-induced mutations in Ha-ras protooncogene and *p53* tumor suppressor gene have been the most extensively studied. Attention has been focused on these genes since mutations in *Ha-ras* and *p53* are frequently observed in DNA derived from skin carcinomas (Daya et al., 1994). The protein coded by *Ha-ras* protooncogene is associated with the cellular membrane and is critical for extracellular stimulation of cellular division (Khosravi-Far and Der, 1994). Several animal studies indicate that activation of *Ha-ras* oncogene is associated with photocarcinogenesis. Strickland et al. (1985) have reported activation of *Ha-ras* by a single treatment of Sencar mice with UVB or PUVA. In addition, Husain et al. (1990) have reported that UVB induces amplification and overexpression of *Ha-ras* protooncogene in mouse skin papillomas and carcinomas. In these animal studies, UV irradiation can definitively be associated with both the formation of morphological changes (i.e., papillomas and carcinomas) and activation of oncogenes(s). Mutations in *Ha-ras* are frequently detected in human skin tumors. Mutations have been reported in up to 45% of human skin squamous cell carcinomas biopsied from sun-exposed areas (Kanjilal et al., 1993; Daya-Grosjean et al., 1993). The incidence of mutations in *Ha-ras* is significantly lower in basal cell carcinomas and melanomas (Kanjilal et al., 1993; Ananthaswamy et al., 1988; Gerrit van der Schroeff et al., 1990).

The *p53* tumor suppressor gene encodes a nuclear phosphoprotein, which plays an important role in the control of cellular proliferation (Levine et al., 1991). The normal or "wild type" *p53* protein acts as a powerful suppressor of cellular growth. Mutations of *p53* gene are frequently observed in animal and human skin tumors. Kress et al. (1992) have found mutations in *p53* gene in up to 50% of squamous cell carcinomas induced by UVB radiation of mice. In addition, all mutations occurred at dipyrimidine sequences and most frequently involved C to T single-base and CC to TT double-base

mutations. As previously discussed, these base substitutions have been found to be characteristic of UVB-induced mutations. Mutations in *p53* gene are also frequently observed in DNA derived from human skin carcinomas. Shea et al. (1992) found overexpression of a mutant *p53* protein in 83% of basal cell carcinomas of the head and neck. In addition, Brash et al. (1991) found mutations of *p53* gene in 58% of human skin cancers. As previously discussed, a detailed analysis of these mutations suggested a distinctive UV-signature in the mutation type. Basset-Seguin et al. (1994) have reviewed evidence that mutation of *p53* may be involved in tumor progression from papilloma to carcinoma, providing further insight into the mechanistic role of *p53* mutations in photocarcinogenesis. Following a review of reports of mutations in *p53* gene, Harris and Hollstein (1993) have suggested that therapy based on the renewal of *p53* function may have future clinical importance. It is clear that application of these techniques of modern molecular biology has resulted in a major leap forward in our understanding of the etiology and possible treatment of photocarcinogenesis.

72.6 CELLULAR MEDIATORS INDUCED BY LIGHT

We have reviewed various sensitized and unsensitized light-induced reactions, such as pyrimidine dimer formation, DNA–protein cross-linking, and various photooxidations. It is still not fully known how these molecular events are involved in the complex physiological processes that give rise to erythema in sunburn or phototoxic reactions. Generally, a UV-induced effect in tissue may be a direct photon effect or may be mediated by diffusible substances induced by photons. Such substances include prostaglandins (PGs), cytokines, histamines, kinins, lysosomal enzymes, and activated oxygen species (e.g., $^1O_2$ and superoxide radical). Research in this field has focused primarily in two areas, prostaglandins and cytokines. PGs (eicosanoids) have been implicated in many physiological processes. The almost ubiquitous occurrence of the PG synthetase enzyme (cyclooxygenase) system and the presence of its substrate fatty acids in membrane phospholipids of mammalian cells suggest that PGs can be formed in most types of cells, where they can act as intracellular messengers (Silver and Smith, 1975).

The role of PGs in cutaneous pathology and inflammation is well established (Goldyne, 1975). PGs were found in whole rat skin homogenates; when the epidermis was separated from the dermis; most of the PG activities were located in the epidermis. The realization that PGs are important in cellular control mechanisms has motivated a great deal of research on their possible role in the etiology of cancer (Snyder and Eaglstein, 1974).

A tentative pathway for PG formation and its interrelation with the adenylate cyclase system in cutaneous tissue after UV irradiation is shown in Figure 72.12. Tissue (specifically membrane) damage, induced by light makes membrane phospholipids "accessible" to the enzyme, phospholipase. This is the first step in inducing the arachidonic acid cascade, which results in PG production (Cohen and Deleo, 1993). In addition the signal transduction mechanism for UV-induced prostaglandin (PGE_2) synthesis has been indicated to involve tyrosine kinases (Miller *et al.,* 1994).

The importance of PGs as mediators of delayed erythema is supported by the observation that inhibitors of PG synthetase such as indomethacin and aspirin can suppress UVB-induced erythema (Snyder and Eaglstein, 1974). However, erythema due to psoralen phototoxicity (PUVA) cannot be suppressed with indomethacin (Morison et al., 1977) and no increase in PG activity is found in exudate from PUVA-inflamed skin (Greaves, 1978). For these reasons, mediators other than PGs are likely to be involved in the pathogenesis of PUVA-induced inflammation.

PGs are rapidly metabolized near the site of their synthesis, which increases the difficulty of studying their role in inflammation. A metabolite of PGE_2, 13,14-dihydro-15-keto-PGE_2 (PGE_2-M), is much more stable and accumulates in plasma, where it can be measured (Tashjian et al., 1977). The introduction of a specific assay for the measurement of PGE_2-M provides an opportunity to examine, in a relatively noninvasive manner, the systemic levels of PGE_2 after a single acute UV injury.

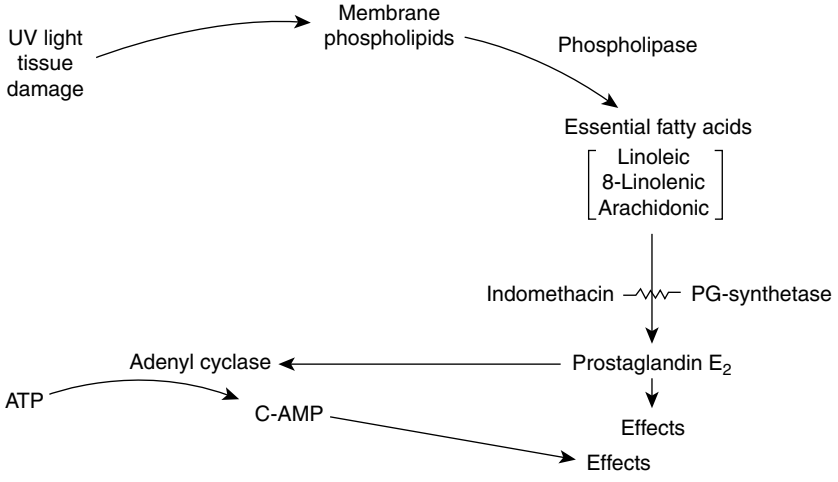


FIGURE 72.12 Tentative pathway of prostaglandin formation and its interrelation with the adenylate cyclase system in cutaneous tissue following UV irradiation.

After exposure to UVB, human suction blister aspirates showed metabolites of arachidonic acid, PGE_2, PGD_2, PGF_{2^-}, and 12-HETE. A contribution of mast cells to UVB-induced inflammation was indicated by the production of PGD_2, as was prostacyclin, a likely derivative of the vascular endothelium (Hruza and Pentland, 1993). UV-light has also been shown to induce increased synthesis of prostaglandins by increasing phospholipase activity in human keratinocyte cultures (Kang-Rotondo et al., 1993), and through increasing the sensitivity of keratinocytes to various inflammatory mediators such as bradykinin (Pentland and Jacobs, 1991). The role of histamine in UV-induced erythema response has also been studied (Gilchrest et al., 1981) and its suggested mechanism of action, by stimulation of prostaglandin release, was reported (Pentland et al., 1990).

The second major area of interest involving cellular mediators is the study of cytokines and their role in cutaneous immune or inflammatory responses. These cytokines include interleukins (IL), hematopoietic colony stimulating factors (CSF), tumor necrosis factors (TNF), and interferons (IFN). Although the role UV light plays in inducing mutagenesis and carcinogenesis is still the focus of much research, the finding that UV light induces the production of cytokines in cell culture or skin (Ansel et al., 1983; Gahring et al., 1984) has resulted in greater attention to the role of these mediators in acute and chronic effects in skin.

Cytokines are small, soluble, polypeptides that are released by a variety of cells including monocytes, macrophages, lymphocytes, fibroblasts, neutrophils, brain cells, and keratinocytes, as well as other cells of the skin. In the skin, cytokines bind to specific cell surface receptors and the signal is transduced by complex protein interactions, such as protein kinase C, to the nucleus where gene expression is altered, thus regulating the proliferation and functions of various target cells or the cytokine-producing cells themselves.

The cytokine environment in *in vitro* experiments may differ substantially from that found *in vivo*. It is important to remember that the biological and pathological responses induced by cytokines depend on the balance between cytokine induction, expression of specific receptors, modulation of cytokine effects by a cascade of cellular events including complex interactions of other cytokines, and by the presence of inhibitors (Di Giovine and Duff, 1990).

A number of studies have suggested that UVB-induced inflammation involving epidermal keratinocytes and dermal fibroblasts results in the synthesis and release of "primary" cytokines, such as interleukin-1α (IL-1α) and tumor necrosis factor α (TNFα), which can stimulate their own production as well as that of a variety of secondary cytokines. Also, IL-1α and TNFα together have been shown to mediate UVB-induced prostaglandin release (Grewe et al., 1993). IL-1, found as two distinct forms (IL-1α and IL-1β) is involved in many biological responses, including cellular proliferation, chemoattraction, and the induction of other cytokines involved in immune regulation, inflammatory responses, growth, and cellular differentiation (Cork et al., 1993).

Epidermal keratinocytes are a major source of IL-1. It is suggested that this large reservoir of IL-1 may provide a protective mechanism against skin injury by its ability to activate a rapid inflammatory response to combat infection and promote wound healing (Gahring et al., 1985). Keratinocytes in normal epidermis also contain inhibitors that are necessary to control the action of IL-1 and other proinflammatory cytokines. If uncontrolled, these cytokines themselves can cause extensive tissue damage. The type I, IL-1 receptor antagonist (IL-1ra) competes with the cytokine for binding to the IL-1 receptor. Soluble type-II inhibitor proteins, shed from cell surfaces, are also present and can bind to a cytokine and prevent it from binding to receptors on target cells. Keratinocytes express only a few high-affinity receptors for IL-1, but the number of these receptors is increased in response to UVB-radiation and trauma. Physical injury or UV-radiation damage to keratinocytes can activate keratinocytes to produce secondary cytokines such as granulocyte macrophage colony stimulating factor (GM-CSF), IL-6, IL-8, macrophage colony stimulating factor (M-CSF), granulocyte colony stimulating factor (G-CSF), and additional IL-1. These cytokines initiate the production of inflammatory infiltrates and activate fibroblasts to proliferate and produce collagen for wound healing. T-cell-derived interferon γ (IFNγ) induces expression of IL-1 receptors, thus increasing the biological effect of the IL-1 released from the keratinocytes (Cork et al., 1993).

TNFα also plays an important role in both humoral and cell-mediated immune and inflammatory responses to infection and injury, including damage induced by UV-light. This cytokine has been found to alter surface properties of endothelial cells, stimulate fibroblasts and neutrophils, and increase production of collagenase and PGE_2. TNFα is a mediator of cachexia, the severe wasting of the body in certain malignant diseases. At high concentrations, TNFα has been shown to play a major role in potentially fatal endotoxic shock. TNFα can also activate polymorphonuclear leukocytes and macrophages, increase their chemotaxis, and stimulate the release of reactive oxygen intermediates including superoxide anion and hydrogen peroxide (Sherry and Cerami, 1988). Significant amounts of TNFα were detected in culture supernatants after normal human keratinocytes and human epidermoid carcinoma cell lines were irradiated by UV-light. After UVB exposure to humans, TNFα was detected in the serum of these volunteers (Köck et al., 1990).

Other important events induced by cytokines that are stimulated or suppressed by UV-light include the expression of surface molecules such as the intercellular adhesion molecule-1 (ICAM-1), which regulates the migration, adhesion, and retention of leukocytes into traumatized sites (Krutmann et al., 1990; Norris et al., 1990; Cornelius et al., 1994).

Epidemiological evidence implicates the contribution of excessive exposure of UV-light on unprotected skin in Caucasians to the great increase in skin cancers that has been reported in recent years. In addition to its carcinogenic effect, UV-radiation has also been found to be immunosuppressive. UVB-induced immunosuppression is one of a

variety of factors that may be a reasonable mechanism for skin cancer. Yoshikawa et al. (1990) reported that susceptibility to tolerance of a topically applied hapten after low-dose UVB exposure to the skin was found only in patients with a proved history of nonmelanoma skin cancer. Shimizu and Streilein (1994) suggest that UVB-radiation converts *trans*- to *cis*-urocanic acid in the epidermis, which in turn causes the production of excessive amounts of TNFα. They reported that the mechanism of UVB-induced tolerance to the hapten dinitrofluorobenzene is separate and distinct from the mechanism that impairs contact sensitivity. Their data suggested that suppressor cells were generated in UV-irradiated mice even though the animals did not display *in vivo* tolerance.

Another significant cytokine, IL-10, has been found to be a suppressive cytokine produced by the TH2 subset of T-helper cells, and keratinocytes, B-cells, mast cells, and monocytes. IL-10 has been shown to have a wide range of activities among which is the ability to block the production of TNFα and IL-1β (Cassatella et al., 1993). IL-10 also blocks natural killer cell stimulatory factor (IL-12), a cytokine of antigen presenting cells and Langerhans cells, and a powerful stimulator of TH1 cells, another subset of helper T cells, to produce IFNγ (D'Andrea et al., 1993). In addition, Langerhans cells exposed to IL-10 failed to cause TH1 cell proliferation and instead induced clonal energy in these cells (Enk et al., 1993). The synthesis of cytokines by TH1 cells is also regulated by the IL-10 cytokine (Mosmann, 1991).

Suppression of delayed-type hypersensitivity was induced in mice after they were injected with supernatants containing IL-10 from UV-irradiated murine keratinocytes. Contrary to the findings of others, no TNFα was detected in the UV-irradiated keratinocyte culture fluid (Rivas and Ullrich, 1992). However, Kang et al. (1994) reported that although human keratinocytes accumulated intracellular IL-10 after *in vivo* exposure of volunteers to four MEDs of UVB, IL-10 was most potently produced and secreted by macrophages. The authors suggested that since macrophages produce IL-10, and immunosuppressive tolerance-inducing macrophages populate the skin after UV exposure, macrophages may play a role in downregulating the UV-induced inflammatory response.

Yoshikawa et al. (1992) studied the effects of TNFα and low-dose UVB on dinitrochlorobenzene (DNCB)-induced contact hypersensitivity (CH). The authors used two strains of mice; those in which UVB-irradiation impaired the induction of CH to DNCB (UVB-susceptible) and those in which UVB-irradiation did not (UVB-resistant). Intradermal injection of TNFα at the ear challenge site before hapten application yielded an amplified CH reaction, even in the UVB-susceptible strain. Anti-TNF~ antibodies given to UVB-susceptible mice neutralized the enhanced CH response to dinitrofluorobenzene (DNFB) but did not affect the CH response of UVB-resistant mice. The results indicated that TNFα, released from UVB-exposed epidermal cells, was a critical mediator of the effects of UVB radiation on both the induction and expression of CH. In addition, these authors found that topically applied DNFB profoundly depleted the epidermis of Langerhans cells, whereas, DNFB applied to UVB-irradiated or TNFα-treated skin was less effective in eliminating these cells. This indicated that TNFα immobilized Langerhans cells transiently within the epidermis. It was proposed that this immobilization had the paradoxical effects of (1) interfering with sensitization (low-dose UVB-irradiation has been found to impair the ability to *induce* CH) by preventing hapten-bearing Langerhans cells from migrating to the draining lymph nodes and, (2) amplifying CH (low-dose UVB-irradiation of previously immunized mice was found to exaggerate the *expression* of CH) by increasing the duration of retention and presentation of the hapten to the epidermis.

The pathological and physiological mechanisms involved in UV-induced immune and inflammatory responses remain poorly understood. Recent advances in this field, however, especially those involving the complex interactions of UV-induced cellular mediators such as prostaglandins and cytokines, have provided a framework of hypotheses to address the important issues concerning these UV-induced reactions.

72.7 PHOTOIMMUNOLOGY

In a broad sense, immunology is the study of how and why the body reacts against anything that is foreign and how an organism can recognize the difference between self and nonself. That UV-light can significantly influence the immune system is a relatively recent discovery. In recent years, exceptional activity, development, and progress in this field has occurred resulting in a new understanding of the connection between light, skin, and the immune system. A complete discussion of these findings is beyond the scope of this section. The interested reader is advised to consult some of the many publications on this subject (Streilein, 1983; Kripke, 1986; Edelson and Fink, 1985; Morison, 1989).

The discipline of photoimmunology began with two important observations: (1) UVB-induced suppression of contact hypersensitivity (CHS) in mice evoked by dinitrochlorobenzene or similar compounds, and (2) UV-light induced alterations in immune functions are involved in the pathogenesis of photocarcinogenesis in mice (Kripke, 1980). UVB-induced tumors in mice are highly antigenic; they are immunologically rejected when transplanted into normal syngeneic recipients, but grow progressively in immunosuppressed animals. Subtumorigenic doses of UVB produce specific systemic alterations, which permit progressive growth of these highly antigenic tumors after transplantation.

The mechanism(s) of these phenomena are still not completely understood. The evidence indicates that UV inhibition of CHS responses and of tumor rejection processes involve suppressor T lymphocytes that inhibit normal immunologic reactions. Recent investigations suggest that the suppressor lymphocytes may be regulatory T cells secreting IL-10 and TGFβ (Schwarz, 2002) or NK T cells (Moodycliffe et al., 2000). UVB-mediated alteration of antigen presenting cells may be a critical event in the generation of suppressor T cells (Beissert et al., 2001). The available evidence suggests that

both CHS responses and tumor rejection processes involve suppressor T lymphocytes that inhibit normal immunologic reactions.

Evidence for the involvement of the immune system in the etiology of photocarcinogenesis in humans is now established. It is possible that chronic exposure to UV causes nonspecific immunosuppression and thus leads to the development of light-induced skin tumors. Long-term clinical treatment with PUVA also induces immunosuppression (Morison et al., 1979), and this may be one of the mechanisms of PUVA-mediated carcinogenesis. The role of UV immunosuppression in melanoma skin cancer is still not established, and has until recently been inaccessible to experimentation. A recent transgeneic mouse model of junctional melanoma initiated by UV irradiation of neonatal mice may rectify this deficit (Noonan et al., 2001).

The immune system involves complex molecular and cellular interactions, which are now being gradually revealed. A major breakthrough, as a result of studies in the fields of photobiology and immunology, has yielded new understanding of the skin as an active element of the immune system. The majority of the cells in the epidermis, Langerhans and Granstein cells, both dendritic populations of the epidermis, and even keratinocytes, have been shown to be active immunologically (Edelson and Fink, 1985). Furthermore, it was shown that certain types of T cells can undergo maturation in the epidermis. To emphasize the connection of these epidermal components to the total immune system, the term skin-associated lymphoid tissue (SALT) has been coined (Streilein, 1983). The concept of immune surveillance, which has undergone ups and downs in its history, has been reformulated: it is presently believed that immune surveillance does exist but is limited to the lymphoreticular and cutaneous systems (Streilein, 1983).

These diverse observations dramatically demonstrate the relevance of photobiology to dermatology and studies of carcinogenesis. An important insight from these findings is the demonstration that both direct (i.e., DNA damage) and indirect (modification of the immune system) effects influence the development of primary skin cancers. Aside from virus-associated cancers, this might be the only experimental carcinogenesis system in which the immune system has been shown to play a role in the carcinogenic process (Kripke, 1986).

Some interesting observations have been made about the mechanism(s) of the photoimmunologic response and the potential mediators involved, including genetic factors (Noonan and Hoffman, 1994). One of the experimental models that has attracted widespread attention is the UVB-suppression of the CHS response in rodents. The CHS method has also been used in human subjects, and UV immunosuppression has been demonstrated (Selgrade et al., 2001). In addition, this phenomenon has also been studied in various *in vitro* systems (CHS is commonly referred to as contact allergy in humans). Mediators produced by keratinocytes exposed to UV may be involved.

Another important approach to this problem was to identify the chromophore responsible for various photoimmunologic responses. However, the identity of the molecular target in the skin for the immunosuppressive effects of UV radiation continues to be controversial. Two schools of thought are currently predominant. The first, elaborated mainly by Kripke et al. (1992), states that DNA is the primary photoreceptor for UV-induced immunological changes. The experimental approach of these authors and of Yarosh et al. (1994), which led to this conclusion, is extremely interesting and inventive. In one set of experiments, after C3H mice were exposed to UV-radiation, T4-endonuclease V, encapsulated in liposomes was used to deliver a dimer-specific excision repair enzyme into the epidermis *in situ*. The fate of the liposome membrane was followed by using a fluorescent, lipophilic dye, and the T4 enzyme was traced by immunogold labeling, followed by fluorescent or transmission electron microscopy. It was found that *in vivo,* liposomes penetrated the stratum corneum where they were localized in the epidermis inside basal keratinocytes (Yarosh et al., 1994). Furthermore, ultrastructural studies demonstrated the presence of liposomes in the cytoplasm of cells in the epidermis. The T4 enzyme was present in both nucleus and cytoplasm of keratinocytes and Langerhans cells. These results confirmed that liposomes could deliver encapsulated enzymes into cells of the skin. In addition, the application of T4 liposomes to UV-irradiated mouse skin decreased the number of cyclobutane pyrimidine dimers in the epidermis and prevented suppression of both delayed and contact hypersensitivity responses. Control, heat-inactivated endonuclease encapsulated in liposomes had no effect. This treatment did not affect immunosuppression induced by 8-MOP plus UVA radiation.

Additional studies by Kripke and Yarosh (1994) involved the marsupial *Monodelphis domestica,* which has an active photorepair enzyme system. The authors demonstrated that both local and systemic types of photoimmunosuppression could be abrogated by exposing the animals to photoreactivating light immediately after UVB irradiation. The photorepair enzyme system is known to be specific for direct removal of cyclobutane pyrimidine dimers.

The studies mentioned suggest that DNA is the photoreceptor in UV-induced immunosuppression and that the primary molecular event mediating this process is the formation of pyrimidine dimers. Furthermore, they illustrate that the delivery of lesion-specific DNA repair enzymes to *in vivo* skin is possible and is an effective tool for restoring immune function and possibly preventing other disorders caused by DNA damage.

The second school of thought concerning the photoreceptor for immunosuppression evolved from the correlation of the action spectrum for UV-induced suppression of CHS with the absorption spectrum of components in the skin, which suggested that urocanic acid (UCA), a molecule present in stratum corneum, may play this role (DeFabo et al., 1981). DeFabo and Noonan (1983) presented additional evidence that the UVB-induced immunosuppression in mice

is initiated by the photoisomerization of UCA. They predicted that the cis-isomer is the natural immunosuppressant, and as such may play an important role as the "mediator" between the environment (UVB) and the immune system. Since that time, additional support for an immunoregulatory role for *cis*-UCA has been provided in a number of experimental systems. Administration of *cis*-UCA *in vivo* has been found to decrease the function of splenic antigen presenting cells (Noonan et al., 1988). Furthermore, topical application of UCA during UV-induced carcinogenesis resulted in an increase in both the tumor number and the degree of malignancy (Reeve et al., 1989). Applying *cis*-UCA to mice without UV irradiation, mimicked the photoimmune suppression effect (Noonan and DeFabo, 1992). In other experiments by Reilly and DeFabo (1991), increasing the UCA levels in mice by feeding its metabolic precursor, histidine, increased the susceptibility to UV suppression in these mice. These findings provide the first evidence that UV-induced immunosuppression can be enhanced by a dietary component (L-histidine).

Research efforts to identify the molecular target for UCA are continuing. It has been shown that *cis*-UCA inhibits induction of cAMP in fibroblasts (Bouscarel, 1998). Most recently evidence has been derived that UCA may in fact act via the neural system in the skin since *cis*-UCA stimulated the release of neuropeptides from sensory neurons (Khalil et al., 2001). This observation would link *cis*-UCA, sensory neurons, neuropeptides, and mast cells in immunosuppression. It has been shown that both histamine and *trans*-UCA upregulate the cAMP formation in human skin fibroblasts in a dose-dependent fashion. *cis*-UCA effectively downregulates this induction. These observations are consistent with the action of UCA via a histaminelike receptor, but the possibility of specific receptors for UCA isomers cannot be excluded. These studies link UCA to a major secondary cell signaling system (Palaszynski et al., 1992). Complementing the role of UCA in these processes, an antibody to *cis*-UCA has been shown to prevent UV-induced immunosuppression (Moodycliffe et al., 1996). Administration of this antibody decreases UV carcinogenesis, consistent with a role for *cis*-UCA immunosuppression in skin cancer.

All these observations strongly suggest that at least two different mechanisms are involved in the UV-modification of the mammalian immune system. The results from the studies on UCA came from experiments performed in *in vitro* systems or in rodents. Although at this time there is no direct evidence that *cis*-UCA is immunosuppressive in humans, the fact that UCA is present in human skin and also isomerizes in response to UVB suggests that it may play the same immunoregulating role in humans. The importance of these findings is increased by the fact that the UV wavelengths (UVB) most affected by depletion of the stratospheric ozone layer are those known to be the most immunosuppressive in animals.

These studies also indicate that light-induced modification of the immune system will be important in the future, with broad applications for managing disorders such as graft rejection, allergies, and autoimmune diseases.

72.8 ALTERATIONS IN GENE EXPRESSION INDUCED BY LIGHT

Any discussion of the cellular and molecular effects of light on the skin is incomplete without reference to the induction of gene expression by light. Through the methods of modern molecular biology, we are now beginning to understand how light can alter the complex program of gene expression within the cell. The discussion to follow will deal with transient changes in gene expression following exposure of cells or skin to light rather than changes in gene expression resulting from mutations and altered cellular phenotype. It will, however, include only a portion of the large body of information on altered gene expression following irradiation that has appeared within the last decade. Recent reviews provide more comprehensive treatments of this subject (Ronai et al., 1994; Holbrook and Fornace, 1991). To date, only alterations in gene expression following UV irradiation in the absence of photosensitizers have been widely studied.

Changes in cellular levels of messenger RNA (mRNA) and related proteins occur within minutes after exposure of cells or skin to UV. The immediate changes following UV exposure have been called the mammalian cell *UV response* (Devary et al., 1992). This immediate UV response involves changes in cellular transcription factors, which are those proteins whose function is to alter the transcription or expression of other cellular genes. The immediate UV response involves two distinct types of changes: (1) modification of preexisting transcription factor proteins, resulting in their increased activities, and (2) resultant elevation of levels of cellular transcription factors through increased transcription of their mRNA. Modification of preexisting transcription factors requires initial UV-induced activation of cytoplasmic proteins including src (Devary et al., 1992), raf-1 and *MAP*-2 (Radler-Pohl et al., 1993) by their phosphorylation. Activated raf-1 leads to the phosphorylation and the resultant activation of the preexisting transcription factor, *c Jun* (Radler-Pohl et al., 1993). Cytoplasmic activation of *c Jun* is an early and essential step in the UV response. Activation of *c Jun* initiates the second phase of the UV response in which levels of transcription factors dramatically increase. The events in this phase of the UV response occur within the nucleus and require formation of mRNA coding for transcription factors including *c Jun* and *c Fos*. Significant increases in the expression of *c Jun* and *c Fos* have been found in cells (Devary et al., 1991), rat skin (Gillardon et al., 1994), and human skin (Roddey et al., 1994) following UV irradiation. The first phase of the UV response, occurring in the cytoplasm, allows a rapid and transient cellular response without mRNA or protein synthesis. The second phase results in a more sustained response to UV irradiation.

Many unanswered questions remain concerning the detailed mechanism connecting UV-induced cellular damage and altered gene expression of transcription factors. There is a strong evidence that UV-induced damage to DNA initiates the UV response (Stein et al., 1989; Yamaizumi and Sugano, 1994). However, as noted, the most immediate changes

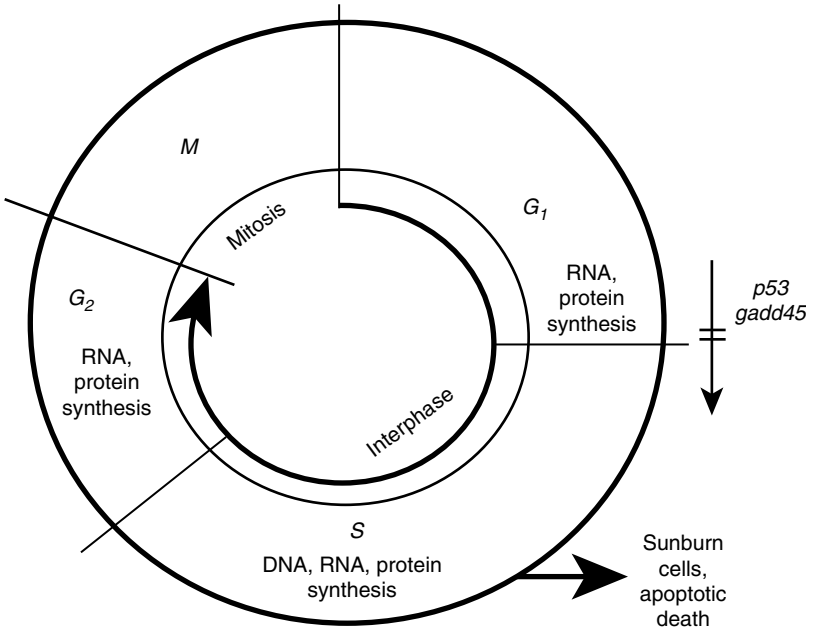


FIGURE 72.13 Diagram of stages in the mammalian cell cycle. Important regulatory stages, or checkpoints, are at the G_1–S and G_2–M transitions. UV radiation induces *p53* and *gadd45*, which are involved with inhibition at the G_1–S transition.

following exposure to UV are cytoplasmic. How UV-induced DNA damage triggers this cytoplasmic response remains to be elucidated and is currently the subject of vigorous research.

The transcription or expression of a wide array of genes is affected by increases in cellular levels of *c Jun* and *c Fos,* which combine to form the heterodimer, activator protein-1 *(AP-1)* (Angel and Karin, 1991). It is *AP-1* that orchestrates the expression of functionally diverse genes within the mammalian cell. AP-1 responsive genes induced by UV radiation include genes associated with protection from oxidative damage, such as metallothioneins I and II (Fornace et al., 1988b), and hemeoxygenase (Keyse and Tyrrell, 1989). AP-1 responsive genes, which play a role in repair of the extracellular matrix in the dermis, such as collagenase (Petersen et al., 1992), are also induced by UV radiation. Ornithine decarboxylase, a central enzyme in polyamine metabolism and stimulation of cell proliferation, is similarly an AP-1 responsive gene induced by UV radiation (Verma et al., 1979).

Many of the important effects induced by UV irradiation of the skin result from alterations in the cell cycle of epidermal cells. These effects include UV-induced hyperplasia and sunburn cell formation (Danno and Horio, 1982). During passage through the cell cycle, the mammalian cell must pass through two critical checkpoints: the G_1–S transition, where DNA synthesis commences; and the G_2–M transition, where mitosis begins (Figure 72.13).

Retardation of progression through these cell cycle checkpoints ostensibly permits repair of UV-damaged DNA before cell division, avoiding propagation of genetic damage. The checkpoint located at the G_1–S transition is critical in assuring that UV-damage is repaired before DNA synthesis and cell division. At least two genes, *p53* and *gadd45,* are involved with arresting the growth of cells containing UV-damaged DNA at this checkpoint in the cell cycle (Kuerbitz et al., 1992; Fornace et al., 1988a). Increases in p53 and gadd45 cellular levels are closely linked to DNA damage in cells (Kastan et al., 1991; Yamaizumi and Sugano, 1994; Fornace et al., 1988a). It was also shown that increased levels of these two proteins result in arrest of growth at the G_1 phase of the cell cycle. Recently, it has been shown that the levels of p53 are dramatically elevated in human skin following moderate levels of UV irradiation. Healy et al. (1994) have shown that suberythemal doses of UVB elicits a significant increase in epidermal p53 levels. Similar results have been reported by Campbell et al. (1993) with UVA, UVB, or UVC. There is now evidence that p53 and gadd45 genes act in concert to arrest cell growth at the G_1 phase. Kastan et al. (1992) provided evidence that p53 acts as a transcription factor whose activity leads to increased expression of gadd45.

72.9 EPILOGUE

Since its beginning around the turn of the century, the science of photobiology has had various stages of development. At a very early stage, through the classical experiments by Raab (1900) and others, it was shown that many dyes and pigments could sensitize various cells and organisms to visible light. The introduction of phototherapy by Niels R. Finsen dates from the same period. These developments extended the boundaries of photobiology to physicists, biologists, and clinicians.

The past three decades marked the beginning of molecular photobiology. An early milestone was the isolation of thymine dimers from living systems exposed to UV. Also, the molecular basis of a genetic disease, xeroderma pigmentosum, was established. The rapid expansion of molecular photobiology significantly contributed to the development of molecular biology and related disciplines and led to the advent of a new clinical discipline, photochemotherapy.

One of the objectives of photobiology will be to shed more light on the relation between phototoxicity and photocarcinogenesis, which is poorly established. It is still believed that chronic phototoxicity can lead to carcinogenesis. In at least a few cases, however, such as chronic phototoxicity evoked by anthracene or porphyrins, no carcinogenic developments were observed. A possible explanation for this phenomenon is that the primary targets of anthracene and other photodynamic sensitizers are molecules not directly involved in the transmission of genetic information.

One of the major conceptual advances in photobiology in the past few years is the perception that photon toxicity is not limited to skin; it can, and often does, induce significant systemic alterations. Therefore, the significance of photobiology exceeds that of dermatology. Basic photobiology should become common knowledge in all branches of basic and clinical medicine.

In all civilizations, humans have worshiped the sun. They have recognized that the sun is the most important of the factors that sustain life on the earth, and that many of our daily rhythms are dependent on the cycles of sunlight. We know today that sunlight is also one of the most potent carcinogens present in the environment. To survive the insult of photons, humans have evolved a group of defense mechanisms. These include keratinization (thickening of the stratum corneum), production of melanin (the most important protective pigment in the skin), and synthesis of urocanic acid (an absorber of UV). The dietary carotenoid pigments also provide some protection by quenching singlet oxygen and various active radical species.

The protective mechanisms evolved against the detrimental effects of the sun are, in a growing number of cases, inadequate because of our modern lifestyles. We must, therefore, increase our understanding of light-induced toxic reactions and judiciously use this knowledge to protect the public health.

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73 Failure of Standard Test Batteries for Detection of Genotoxic Activity of Some Chemicals Used in Dermatological and Cosmetic Products

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73.1 INTRODUCTION

Guidelines for assessment of the genotoxic potential of chemicals are based on the premise that DNA damage and its fixation in the form of gene mutation and chromosomal damage is generally considered to be essential in the multi-step process of carcinogenesis, even if genetic changes may play only a part in this complex process. The standard 3-test battery for the genotoxicity testing of pharmaceuticals [1] consists of: (1) a test for gene mutation in bacteria; (2) an *in vitro* test with cytogenetic evaluation of chromosomal damage in mammalian cells or an *in vitro* mouse lymphoma *tk* assay; and (3) an *in vivo* test for chromosomal damage using rodent hematopoietic cells. The recommended genotoxicity tests for cosmetic ingredients [2] are: (1) a bacterial test for gene mutation; (2) an *in vitro* test for clastogenicity and aneuploidy (metaphase analysis or micronucleus test); and (3) an *in vitro* mammalian cell mutation assay (mouse lymphoma assay as the preferred choice); further *in vivo* testing may be justified when concern is raised over positive results in *in vitro* tests.

In the last year [3], the performance of a battery of three of the most commonly used *in vitro* genotoxicity test—Ames + mouse lymphoma assay + *in vitro* micronucleus or chromosomal aberrations test—has been evaluated for its ability to discriminate rodent carcinogens and noncarcinogens from a large data base of over 700 chemicals. Of the 533 carcinogens with valid genotoxicity data, 93% gave positive results in at least one of the three tests; only 19 carcinogens, out of 206 tested in all three tests, gave consistently negative results in the full 3-test battery. On the basis of this evaluation, the European Scientific Committee for Cosmetics and Non-Food Products (ESCCNFP) has reviewed the guidelines for testing hair dyes for genotoxicity [4]. This battery of six *in vitro* tests—bacterial reverse mutation (Ames) test, *in vitro* mammalian chromosome aberration test, *in vitro* mammalian cell mutation test, DNA damage and repair (UDS) test in mammalian cells *in vitro*, *in vitro* mammalian micronucleus test, *in vitro* Syrian Hamster Embryo (SHE) cell transformation test—differs substantially from the batteries of two or three *in vitro* tests recommended in other guidelines. After evaluation of the types of chemicals used in hair dyes and comparison with other guidelines for testing a wide range of chemicals, the ESCCNFP concluded that the potential genotoxic activity of hair dyes may effectively be determined by the application of the three *in vitro* tests recommended for the genotoxicity testing of the other cosmetic ingredients [2]; that is, by the same battery of three of the most commonly used *in vitro* genotoxicity tests judged by Kirkland et al. [3] as a useful tool to identify chemicals possessing carcinogenic or noncarcinogenic potential.

Recently, we have put in evidence [5] that there are some chemicals, classified by the International Agency for Research on Cancer (IARC) as probably or possibly carcinogenic to humans on the basis of a sufficient evidence for carcinogenicity in experimental animals, which gave consistent negative results in the test battery for the genotoxicity testing of pharmaceuticals, and in contrast provided positive results in other nonroutinely employed genotoxicity assays. Therefore, it cannot be taken for granted that with this 3-test battery the risk of false negative results for compounds with genotoxic/carcinogenic potential is completely avoided.

The aim of this review is to verify whether a series of chemicals used in dermatological preparations or in cosmetics, which have been found to be carcinogenic in at least one sex of mice or rats, are identified by the 3-(*in vitro* and *in vivo*) test battery for genotoxicity testing of pharmaceuticals [1], and by the 3-(*in vitro*) test battery for the gentoxicity testing of hair dyes and of the other ingredients of cosmetics[2,4].

73.2 EXAMPLES OF DRUGS USED IN DERMATOLOGY THAT TESTED POSITIVE FOR CARCINOGENICITY AND NEGATIVE FOR GENOTOXICITY

The Physicians' Desk Reference [6] publishes for each drug the results of short-term genotoxicity/mutagenicity/clastogenicity assays and of long-term carcinogenicity assays available. Table 73.1 lists, for each of the drugs contained in dermatological preparations considered in this review, the results of the following assays: long-term carcinogenicity assays in mice and rats; *in vitro* forward and reverse mutation in *Salmonella typhimurium* and other bacteria; *in vitro* gene mutation, sister chromatid exchanges (SCE), and chromosomal aberrations (CA) in animal and human cells; *in vivo* SCE, CA, and micronucleus (MN) formation in hematopoietic rodent and human cells. Each drug is then considered separately providing details on the carcinogenesis and genotoxicity assays performed.

Adapalene [6], which binds to nuclear retinoid receptors and is indicated for the topical treatment of acne vulgaris, when administered in rats by the oral route at doses of 0.15–0.5–1.5 mg/kg/day, i.e., up to six times in terms of mg/m^2/day the topical maximum recommended human dose (MRHD), was found to increase the incidence of thyroid follicular-cell adenomas and carcinomas in females, and of benign and malignant pheochromocytomas in the adrenal medulla of males. In a series of *in vitro* and *in vivo* studies—Ames test, mouse lymphoma assay, Chinese hamster ovary cell chromosomal aberrations assay, and mouse micronucleus test—adapalene did not exhibit genotoxic effects.

Fluconazole [6], a synthetic triazole antifungal agent used topically in the treatment of mycosis, was found to increase the incidence of hepatocellular adenomas in male rats treated with 5 and 10 mg/kg/day (i.e., up to seven times the MRHD). With or without metabolic activation, fluconazole was negative in tests for mutagenicity in four strains of *S. typhimurium* and in the mouse lymphoma assay; no evidence of chromosomal aberrations was obtained *in vitro* in human lymphocytes exposed to 1 mg/mL of fluconazole, and *in vivo* in murine bone marrow cells of mice treated by the oral route.

Imiquimod [6], an immune response modifier indicated for the topical treatment of actinic keratosis, when applied to the backs of mice three times a week for 24 months up to a dose of 5 mg/kg (251 × MRHD) produced a statistically significant increase in the incidence of liver adenomas and carcinomas in males. In contrast, no evidence of mutagenic or clastogenic potential was observed in five *in vitro* assays (Ames assay, mouse lymphoma assay, Chinese hamster ovary cell chromosomal aberrations assay, human lymphocyte chromosomal aberrations assay, and SHE cell transformation assay), or in three *in vivo* assays (rat and hamster bone marrow cytogenetics assay and a mouse dominant lethal test).

Pimecrolimus [6], a chloro-derivative of the macrolactam ascomycin indicated for the topical treatment of atopic dermatitis, in a 2-year rat dermal carcinogenicity study was found to produce a statistically significant increase in the incidence of follicular cell adenomas of the thyroid in male

TABLE 73.1
Information Provided by the Standard 3-Test Battery on the Genotoxicity of Some Drugs of Dermatological Use Which are Carcinogenic in Rodents

	Carcinogenicity in Rodents		Gene Mutation	Mammalian	Cells	*In Vitro*		*In Vivo*	
Drug	Mouse	Rat	in Bacteria	Gene Mutation	SCE	CA	SCE	CA	MN
Adapalene	nd	+	—	—	nd	—	nd	nd	—
Fluconazole	nd	+	—	—	nd	—	nd	—	nd
Imiquimod	+	nd	—	—	nd	—	nd	—	nd
Pimecrolimus	+	+	—	—	nd	—	nd	nd	—
Tacrolimus	+	nd	—	—	nd	nd	nd	—	nd
Terbinafine	nd	+	—	—	—	—	nd	—	—
Tretinoin	+	nd	—	nd	nd	nd	nd	nd	—

Note: The data indicate for each assay the negative response as reported in the Physicians' Desk Reference [6]. The abbreviations are: SCE, sister chromatid exchanges; CA, chromosomal aberrations; MN, micronucleus; nd, not determined.

animals at a dose of 2 mg/kg/day (1.5 × MRHD). In a 13-week mouse dermal carcinogenicity study, lymphoproliferative changes including lymphomas, were observed at a dose of 25 mg/kg/day (47 × MRHD). In a mouse oral carcinogenicity study, a statistically significant increase in the incidence of lymphomas was noted in both males and females at a dose of 45 mg/kg/day (258–340 × MRHD). In another rat oral carcinogenicity study, the incidence of benign thymomas was increased in males at a dose of 5 mg/kg/day and in females at a dose of 10 mg/kg/day. A battery of *in vitro* tests, including the Ames assay, mouse lymphoma assay, the chromosomal aberrations assay in V79 Chinese hamster cells, and an *in vivo* mouse micronucleus test, revealed no evidence for mutagenic or clastogenic potential of pimecrolimus.

Tacrolimus [6], used for the prophylaxis of organ rejection and in atopic dermatitis, did not increase tumor incidence in mice and rats when administered orally. However, in a 104-week dermal carcinogenicity study performed in mice with tacrolimus ointment (0.03–3%) a statistically significant increase in the incidence of pleomorphic lymphomas was observed in mice of both sexes treated with 0.1% (3.5 mg/kg) ointment, as well as undifferentiated lymphomas in females treated with the same dose. In contrast, no evidence of genotoxicity was seen in the following assays: *S. typhimurium* and *Escherichia coli* mutagenicity assays, Chinese hamster ovary and Chinese hamster lung cell mutagenicity assays, *in vivo* clastogenicity assay in mice, and unscheduled DNA synthesis in rodent hepatocytes.

Terbinafine [6], which is indicated for the topical treatment of onychomycosis, was found to increase the incidence of liver tumors in male rats given a daily dose of 62 mg/kg (2 × MRHD). In contrast, terbinafine did not induce mutations in *S. tphimurium* and *E. coli*, was nonmutagenic in Chinese hamster fibroblasts, and did not increase the frequencies of SCE and chromosomal aberrations in Chinese hamster lung cell. *In vivo* it gave negative responses in Chinese hamsters for chromosomal aberrations and in the mouse micronucleus test.

Tretinoin [6], a retinoid metabolite of vitamin A indicated for the topical treatment of acne vulgaris, in a 91-week dermal carcinogenicity study in which CD-1 mice were administered with 0.017% and 0.035% formulations, was found to induce cutaneous squamous cell carcinomas and papillomas in the treatment area of some females. A dose-related incidence of liver tumors was observed at the same concentrations in male mice. These doses (0.5–1 mg/kg/day) are 10–20 times the maximum human systemic dose. Moreover, studies in hairless albino mice suggested that concurrent exposure to tretinoin might enhance the tumorgenic potential of carcinogenic levels of UVB and UVA. In contrast, tretinoin was found to give negative responses in the Ames test and in the *in vivo* mouse micronucleus assay.

A further example of the failure of the standard 3-test battery in detecting the genotoxic activity of carcinogens is provided by permethrin [6], which was found to be carcinogenic in mice yet gave negative responses in a battery of nonspecified *in vitro* and *in vivo* genotoxicity assays.

Finally, it is worth noting that a drug found to be noncarcinogenic in rodents can test positive in genotoxicity assays; an example being *acyclovir* [6] found to be noncarcinogenic in lifetime assays in mice and rats, yet positive in 5 of 16 *in vitro* and *in vivo* genotoxicity assays.

TABLE 73.2
Information Provided by the Standard 3-Test Batteries on the Genotoxicity of Some Compounds Used in Cosmetic

		Carcinogenicity in Rodents			Mammalian Cells *In Vitro*				*In Vivo*		
Compound	IARC Evaluation	Mouse	Rat	Gene Mutation in Bacteria	Gene Mutation	SCE	CA	MN	SCE	CA	MN
Butylated hydroxyanisole	2B	nd	+	3−	2−	1−	2−/1+	1+	nd	nd	nd
Chlorodiflouro-methane	3	nd	+	1(+)	2−	nd	nd	nd	nd	2−	nd
D&C Red No. 9	3	nd	+	3−/ 1(+)	1−	1−	1−	nd	nd	nd	1−
p-Dimethylamino-azobenzene	2B	+	+	1−/1+	1−	nd	1+	nd	nd	nd	nd
1,4- Dioxane	2B	+	+	5−	2−	1−/1(+)	2−	1−	nd	nd	3−/1 ?/1+
HC Blue No 1 (purified)	2B	+	+	3−	2−	1−/1 +	1−	nd	nd	nd	3−/1+
Lead acetate	2B	nd	+	3−	nd	1−	3−/1 ?/2+	nd	nd	1−/2+	1−
Phenacetin	2A	+	+	5−/ 2+	1 ?	nd	1−/1+	1−	1(+)	1(+)	2−/1+
Titanium dioxide	3	nd	+	2−	1−	nd	1−	1−	nd	nd	nd

Note: The data indicate for each assay the number of negative [−] inconclusive [?] weakly positive [(+)], and positive [+] responses as reported in IARC Monographs on the "Evaluation of Carcinogenic Risks to Human" and in peer-reviewed journals. The abbreviations are: SCE, sister chromatid exchanges; CA, chromosomal aberrations; MN, micronucleus; nd, not determined.

73.3 EXAMPLES OF CHEMICALS USED IN COSMETICS THAT TESTED POSITIVE FOR CARCINOGENICITY AND SUBSTANTIALLY NEGATIVE FOR GENOTOXICITY

The *IARC Monographs* on the "Evaluation of Carcinogenic Risks to Humans" publish the results of all short-term genotoxicity/mutagenicity/clastogenicity assays and of all long-term carcinogenicity assays available. Table 73.2 lists, for each of the chemicals used in cosmetics considered in this review, the results provided by the *IARC Monographs* and by other sources of the following genotoxicity tests: *in vitro* forward and reverse mutation in *S. typhimurium* and other bacteria; *in vitro* gene mutation, SCE, CA, and MN in animal and human cells; *in vivo* SCE, CA, and MN formation in hematopoietic rodent and human cells. Taking into account that in some cases the same test was performed in more than one study and the results may be discordant presumably because of differences in the protocol for each test, the number of positive, weakly positive, inconclusive, and negative responses are listed. Each compound is then considered separately to show results obtained in genotoxicity assays, which differ for the end point examined or for the target cells from those listed earlier.

Butylated hydroxyanisole [7], present as a preservative in various skin products, was found to produce benign and malignant tumors of the forestomach in rats and hamsters when administered in the diet. No data are available to evaluate its carcinogenicity to humans. On the basis of sufficient evidence for carcinogenicity in experimental animals, the IARC classified butylated hydroxyanisole as possibly carcinogenic to humans (Group 2B) [8]. Results provided by the *in vitro* standard 3-test battery [7] were all negative; butylated hydroxyanisole was nonmutagenic to *S. typhimurium* in both *in vitro* and in a host mediated assay, did not induce 6-thioguanine-resistant mutants in cultured Chinese hamster ovary cells and V79 cells, and did not increase the frequency of SCE or chromosomal aberrations in Chinese hamster CHL cells on in Chinese hamster DON cells, respectively. However, according to Kirkland et al. [3] BHA was found to give a positive *in vitro* response in the micronucleus test and in the chromosomal aberrations test. Sex-linked recessive lethal mutations were not induced in *Drosophila melanogaster*. It is worth noting that butylated hydroxyanisole has been found to be a strong inducer of oxidative DNA damage in the epithelial cell of the rat glandular stomach [9].

Chlorodifluoromethane [10], until recently used as propellant in hair sprays, was found to increase the incidence of fibrosarcomas and Zymbal-gland tumors in male rats in an inhalation study. No data are available for evaluating its carcinogenicity to humans. On the basis of limited evidence for carcinogenicity in experimental animals, the IARC [8] judged chlorodifluoromethane nonclassifiable regarding its carcinogenicity to humans (Group 3). Results provided by genotoxicity tests were substantially negative [10–12]. Chlorodifluoromethane was found to be weakly mutagenic in *S. typhimurium*, but did not induce 6-thioguanine resistant mutants in Chinese hamster V79 cells and reverse mutation at the *hprt* locus in Chinese hamster ovary cells. Neither did it increase the frequency of chromosomal aberrations in rat and murine bone marrow cells. Moreover, chlorodifluoromethane did not induce either mutation or gene conversion in *Saccharomyces cerevisiae*, forward mutation in *Saccharomyces pombe*, or DNA repair in transformed human cells. It also gave a negative response in the dominant lethal test in rats and mice, and in a host-mediated assay with microbial cells.

D&C Red No. 9 [13], a grade of CI Pigment Red 53:1 used in temporary hair dye formulations and in some countries as a lipstick colorant, was found to produce splenic sarcomas in male rats, and to increase the incidence of neoplastic liver nodules in animals of each sex. No data are available to evaluate its carcinogenicity to humans. On the basis of limited evidence for carcinogenicity in experimental animals, D&C Red No. 9 was judged by the IARC nonclassifiable regarding its carcinogenicity to humans (Group 3). D&C Red No. 9 was inactive in all the assays of the 3-test batteries in which it was examined. It was not mutagenic in *S. typhimurium* with the exception of a weakly positive response in the TA98 strain in the presence of a precipitate. D&C Red No. 9 did not induce mutation at the *tk* locus in the mouse lymphoma assay, or SCE or chromosomal aberrations in Chinese hamster ovary cells. After oral administration it did not cause micronucleus formation in rat bone marrow cells. Moreover, it induced DNA repair synthesis neither in primary rat hepatocytes nor in the liver of intact rats. Whereas, the genotoxic nature of D&C Red No. 9 has been put in evidence from its incubation with a rat cecal preparation under anoxic conditions to reduce the azo bond. The presumed major reduction product, 1-amino-2-naphthol, was mutagenic in the *S. typhimurium* TA 100 strain [14].

p-Dimethylaminoazobenzene [15], contained in brillantines, was found to produce liver tumors in rats by several routes of administration as well as in newborn mice, and bladder tumors in dogs given orally. No data are available to evaluate its carcinogenicity to humans. The IARC [8] classified *p*-dimethylaminoazobenzene as possibly carcinogenic to humans (group 2 B) on the basis of sufficient evidence for carcinogenicity in experimental animals. According to the IARC [15] *p*-dimethylaminoazobenzene did not induce reverse mutations in *S. typhimurium* TA1538 in the presence of rat liver microsomal systems. However, they were produced in the same strain by a urinary metabolite of rats fed this chemical. It was non-mutagenic in *Drosophila melanogaster*. However, according to Kirkland et al. [3] it was found to test positive in the Ames assay and for clastogenic activity in cultured mammalian cells, but tested negative in the mouse lymphoma assay. In rats the intraperitoneal injection of tritium-labeled *p*-dimethylaminoazobenzene gave rise to DNA adducts in liver and spleen [15].

1,4-Dioxane [16], a common trace component of cosmetic products such as shampoos and skin conditioners, was found to produce, when administered orally, an increased incidence of hepatocellular adenomas and carcinomas in

mice,; tumors of the nasal cavity, hepatocellular adenomas and carcinomas; mesotheliomas of peritoneum; and subcutaneous fibromas, mammary adenomas, and fibroadenomas in rats. By i.p. injection, it produced lung tumors in mice. Death from cancer was not elevated in a single, small prospective study of workers exposed to low concentrations of 1,4-dioxane. The IARC classified 1,4-dioxane as possibly carcinogenic (Group 2B) to humans on the basis of inadequate evidence for carcinogenicity in humans and sufficient evidence in experimental animals. Most tests for genotoxic activity produced negative results; 1,4-dioxane was not mutagenic in *S. typhimurium*, did not induce gene mutation at the *tk* locus in mouse lymphoma L5178Y cells or chromosomal aberrations in Chinese hamster ovary cells *in vitro*. A positive result was obtained in only one of five assays in mouse bone marrow cell for micronucleus induction. Moreover, 1,4-dioxane did not induce sex-linked recessive lethal mutations in *Drosophila melanogaster*, nor DNA repair in primary rat hepatocytes or in the liver of intact rats[A4], and also did not bind to DNA of rat liver cells. However, 1,4-dioxane was found to induce DNA fragmentation in primary rat hepatocytes and in the liver of intact rats, SCE in Chinese hamster ovary cells, and transformation of BALB/c 3T3 mouse cells. In a subsequent study of Morita and Hayashi [17] 1,4-dioxane tested negative in the bacterial reverse mutation assay and in the mouse lymphoma assay. It did not induce chromosomal aberrations, SCE and micronucleus formation in Chinese hamster ovary cells, but was positive in the mouse liver micronucleus test.

HC Blue No. 1 [13], a semi-permanent hair dye, administered up to 6000 ppm in the diet, was found to produce hepatocellular adenomas and carcinomas in mice of each sex and to increase the incidence of thyroid follicular-cell adenomas in males. An increased incidence of pulmonary adenomas and carcinomas was seen in female but not in male rats. No data are available to evaluate its carcinogenicity to humans. It is classified by the IARC as possibly carcinogenic to humans (Group 2B). Results provided by the standard 3-test batteries were substantially negative. Purified HC Blue No. 1 was not mutagenic in bacteria; did not bind to DNA of the *S. typhimurium* strain TA98 *in vitro*; did not induce gene mutation at the *hprt* locus in Chinese hamster lung V79 cells or at the *tk* locus in mouse lymphoma L5178Y cells. It increased SCE frequency in one of two assays on Chinese hamster ovary cells but not the frequency in the same cells of chromosomal aberrations: *In vivo*, a positive response in the mouse micronucleus test was seen in female ICR mice but not in males of the same strain or in CBA and CD-1 mice. In contrast, it elicited DNA repair synthesis in primary cultures of hepatocytes from mice, rats, hamsters, rabbits, and monkeys, but not in human HeLa cells. It is worth noting that commercial nonpurified HC Blue No. 1 preparations were substantially positive in the standard 3-test battery.

Lead acetate [18], contained in temporary hair dyes, was found to produce benign and malignant tumors of the kidney in rats following oral or parenteral administration, and gliomas in rats treated by the oral route. Epidemiological data are considered inadequate. The IARC [8] classified inorganic lead compounds as possibly carcinogenic to humans (Group 2B) on the basis of sufficient evidence for carcinogenicity in animals. Lead acetate was not mutagenic in bacteria either *in vitro* or in a host-mediated assay. There are conflicting reports on the effect of lead acetate on chromosomal aberrations in cultured mammalian cells and in rodents, but any increase of the frequency of micronuclei in bone marrow cells was absent. It tested negative in *Saccharomyces cerevisiae* for mitotic recombination.

Phenacetin [8,19], present in bleaching solutions and in permanent-wave preparations, was found to produce benign and malignant tumors of the urinary tract in mice and rats, and of the nasal cavity in rats when given orally. Evidence for carcinogenicity to humans is considered limited. The IARC [8] classified phenacetin as probably carcinogenic to humans (Group 2A) on the basis of sufficient evidence for carcinogenicity to animals. According to IARC [8,12,19], phenacetin was not mutagenic to bacteria in several assays and resulted positive only when tested in the presence of a metabolic system derived from hamster liver (not from mouse or rat liver). It was negative in a host-mediated assay. Phenectin did not induce recessive lethal mutation in *Drosophila melanogaster* or DNA fragmentation in cultured mammalian cells. It produced a positive result in an *in vitro* chromosomal aberration test in Chinese hamster cells in the presence, but not in the absence of metabolic activation. The results of studies on the induction of chromosomal aberrations, SCE and micronucleus in rodents treated with phenacetin *in vivo* were contradicting. According to Kirkland et al. [3], phenacetin tested negative for micronucleus formation *in vitro*, and equivocal in the mouse lymphoma assay. It was found positive [20] for DNA damage in the rat kidney.

Titanium dioxide [21], contained in skin and nail products, was found to produce an increased incidence of lung adenomas in rats of both sexes, and squamous-cell carcinomas in females. The only available epidemiological study provided inconclusive results. On the basis of limited evidence for carcinogenicity in humans, the IARC judged titanium dioxide nonclassifable as to its carcinogenicity in humans (Group 3). According to the IARC titanium dioxide was nonmutagenic in *S. typhimurium* strains TA98, TA100, TA1535, TA1537, TA1538, and in *E. coli* WP2 *uvrA*. Moreover it did not induce cell transformation. According to Kirkland et al. [3] titanium dioxide tested negative not only for bacterial mutagenicity but also in the mouse lymphoma assay, and for chromosomal aberrations and micronucleus formation in cultured mammalian cells.

73.4 CAUSES OF FALSE NEGATIVE RESULTS

In vitro and *in vivo* short-term genotoxicity assays have been developed to enable the detection of genotoxic agents, and a battery of such tests should ensure that a chemical, which could potentially induce cancer in humans, does not escape the preliminary phase of screening. Unfortunately, it has been ascertained, and is now generally recognized, that none of the available short-term tests are capable of detecting all genotoxic

chemicals [5,22]. It is therefore useful to examine separately the causes of false negative results in both *in vitro* and *in vivo* tests. As indicated previously for the genotoxicity testing of pharmaceuticals an *in vivo* test is also recommended [1], whereas the genotoxicity testing of hair dyes and other cosmetic ingredients usually only requires *in vitro* tests [2,4] to be performed.

73.4.1 *In Vitro* Assays

Most carcinogens require biotransformation to DNA reactive species to exert a genotoxic effect, but in both bacteria and mammalian cells that are routinely employed as targets for *in vitro* tests, the enzyme systems involved in the metabolism of xenobiotics are either lacking or expressed to a limited extent. To circumvent this obstacle, tests are carried out in both the absence and presence of an exogenous metabolic system—usually the rat liver S9-mix derived from animals pretreated with inducers such as phenobarbitone, β-naphtoflavone, or Aroclor 1254—but this metabolic system should only be considered a first approximation of what occurs in mammalian cells and in the intact animal [23]. Other complicating factors may be the qualitative and quantitative differences in the biotransformation of chemicals in different cell types and in cells from different species [24,25]. In assays comprising rat liver S9 preparations only, interspecies differences in the metabolism of xenobiotics should not be overlooked, since the animal species from which the liver S9 is obtained may be determinant for the efficient detection of carcinogens as mutagens [26]. Taking into account that the aim of the assays is the assessment of human risk, differences in metabolism between rodents and humans are of fundamental importance. Comparison of mutagenicity data obtained with human liver preparations with those obtained with rat liver preparations showed great interspecies differences in the capacity to activate certain chemicals [27,28], and a large inter-individual diversity in the mutagenic response to mutagens of human S9 fractions [28,29]. As an example of fact, the rat and human P450 enzymes can differ in their substrate selectivity and reactions catalyzed; in particular the CYP2 family demonstrates vast differences in metabolism between rats and humans. Other possible causes of a false negative result include: metabolism at high doses may be qualitatively and quantitatively different from that occurring at pharmacologically relevant doses, due to test compound-induced inhibition of the formation of down stream metabolism or competition with metabolites for further metabolism; dimethyl sulfoxide, often used as solvent in *in vitro* tests, inhibits several P450-mediated reactions even at low concentrations [30]; finally, it must be considered that some carcinogens are activated to DNA-damaging species of to short half-life that they can react only with the DNA of the cell in which they are formed [31]. It cannot be excluded that this may happen, for example, in epidermal cells.

It is generally accepted that none of the *in vitro* short-term tests can detect all genotoxic carcinogens. The Ames test has been found to poorly detect carbamyls and thiocarbamyls, phenyls, benzodioxoles, polychlorinated alipathic, cyclic and aromatic hydrocarbons, steroids, antimetabolites, and symmetrical hydrazines [32]. The 10% of rat carcinogens with structural alert are nonmutagenic in *S. typhimurium* [33]. An analysis of data on the clastogenicity of 951 chemicals, revealed that 26 out of 111 sufficient positive carcinogens were evaluated as negative, some of them being genotoxic and with structural alerts [34]. The mouse lymphoma assay that is capable of detecting chemicals acting as point mutagens, as well as those causing some types of chromosomal aberrations, evaluated as negative 24 of 107 chemicals classified as sufficient positive carcinogens (some of which were also in this case genotoxic [35]). Similarly, some carcinogens have been found to be nonmutagenic to V79 cells at the *hprt* or the Na^+/K^+ ATPase locus [36].

73.4.2 *In Vivo* Assays

In the *in vivo* test of chromosomal damage in rodent hematopoietic cells, indicated by the guidelines for genotoxicity testing of pharmaceuticals (whereas in genotoxicity testing of cosmetic ingredients it might be justified only by positive results in *in vitro* tests), the occurrence of a false negative result may be caused by; the pharmacokinetic behavior of the test compound; and a very high dose may inhibit enzyme systems involved in its metabolic activation. Owing to an unequal distribution in the tissues of the body and to differences in the activation/detoxification potential of the various tissues, there are chemicals, which induce a significant genotoxic and carcinogenic effects in only one organ or cell type. Evidence of a genotoxic tissue-specific effect has been observed in rats, with some chemicals carcinogenic in the kidney [20,37], the urinary bladder [38], and the thyroid [39], which themselves had previously been found to give contradictory or false negative results in both the *in vitro* and *in vivo* standard batteries of genotoxicity tests. Certainly it cannot be excluded for a substance applied topically to the skin the occurrence of a skin-specific genotoxic effect that would otherwise be undetected in the cytogenetic evaluation of chromosomal damage in bone marrow cells.

In fact, the bone marrow hematopoietic cells, which are the target in the *in vivo* assays indicated in the 3-test standard battery for genotoxicity testing of pharmaceuticals, have a low biotransformation capacity, and reactive species of short half-life produced in the liver or in other organs may be unable to reach them. Chemicals not easily detected by the bone marrow micronucleus test are aromatic amines, *N*-nitroso compounds, nitroimidazoles, and haloalkanes [40]. According to Morita et al. [41], the mouse erythrocyte micronucleus assay detects only 52% of chemicals classified by the IARC as carcinogenic (Group 1), probably carcinogenic (Group 2A), and possibly carcinogenic (Group 2B) to humans. Finally it should be considered that the type of response—positive or negative—provided by a short-term *in vivo* assay may depend on the species, strain, and sex of the animal used, as it was found to occur for carcinogenic activity. Furthermore, an analysis of purchase [42] revealed that 43 of 250 carcinogens were active in mice alone or in rats alone, and the carcinogenic

potency database of Gold et al. [43] demonstrated that many carcinogens are not only species-specific, but also strain- and sex-specific. Taking into account that short-term *in vivo* assays usually employ mice or rats, and that the phylogenetic difference between humans and these rodents is undoubtedly greater than that between mice and rats, it is evident that extrapolation to humans of results provided by these assays may be subjected to substantial errors.

73.5 DISCUSSION AND CONCLUSIONS

As exemplified and discussed in this review, the routinely employed standard test batteries, even if capable of detecting the large majority of genotoxic carcinogens, fails to detect some of them. This limit has been clearly confirmed by an analysis of results provided by *in vitro* genotoxicity tests performed in Germany [22]. This analysis indicates that 72.2% of chemicals found positive in the bacterial gene mutation assay were negative in both gene mutation and chromosomal aberration assays in mammalian cells, and more than 80% of the *in vitro* clastogens were found negative in the bacterial mutation assay. A similar picture is given by our analysis of the results provided by the standard test batteries for some compounds used in dermatology and in cosmetic which have been found to be carcinogenic in at last one sex of mice or rats. All the seven drugs used in dermatology tested negative in all the genotoxicity assays in which they were examined (Table 73.1). Five of them were tested *in vitro* for mutagenic activity in bacteria and for gene mutation and chromosomal aberrations in cultured mammalian cells. Of the other two drugs, one was tested for mutagenic activity in both bacteria and mammalian cells, and one only with the Ames test. All the seven drugs were examined *in vivo* for chromosomal aberrations or micronucleus formation in bone marrow cells. For these drugs no clear evidence for a nongenotoxic mechanism exists and no information is available to definitely establish whether these drugs are genotoxic or epigenetic carcinogens. However, the negative results provided by the standard test batteries do not exclude a genotoxic mechanism of action that may be revealed by assays other than those included in these batteries. Concerning the nine carcinogenic compounds used in cosmetics (Table 73.2), the results provided by the standard test batteries, even if to some extent discordant, when taken as a whole, confirm the possibility that these batteries may fail in identifying some genotoxic carcinogens. All the nine compounds were tested *in vitro* for mutagenic activity in bacteria and for gene mutation and clastogenic activity in mammalian cells. Some tests were repeated in different studies and in some case the results are discordant, but the large majority of them were negative. Thus, the conclusion is supported that all the nine compounds are substantially identified as nongenotoxic by the standard *in vitro* battery. With respect to the *in vivo* genotoxicity tests, two of the six compounds examined in at least one type of assay gave a negative response, and the other four, as in the *in vitro* tests, gave discordant results. Results of *in vivo* tests were not available for butylated hydroxyanisole, *p*-dimethylaminoazobenzene, or titanium dioxide. It is worth noting that of the nine compounds used in cosmetic at least seven were identified as genotoxic by assays not included in the standard batteries, or should be considered genotoxic on the basis of their biotransformation.

Since the standard batteries of genotoxicity tests are unable to identify all genotoxic carcinogens, for at least some of which that give negative responses in these batteries, performing other types of genotoxicity assays may be considered a wise decision. This opportunity has been already anticipated by the IPCS harmonization of methods for the prediction and quantification of human carcinogenic/mutagenic hazard that stated, "minimum criteria testing schemes should not be invoked to prevent the conduct of appropriate, albeit non-routine, assays on chemicals" [44]. Obviously it is problematic to establish what chemicals deserve further testing, and how many tests should be performed before a chemical is classified as definitely nongenotoxic. Several tests that could be used for additional investigations of the possible genotoxic activity have been indicated in a previous review [5]. They include the detection of covalent DNA adducts, DNA damage and DNA repair either in various types of cultured mammalian cells from animals and human donors, or *in vivo* in multiple organs of mice or rats. Concerning those chemicals contained in pharmaceutical preparations used in dermatology as well as in cosmetics, it should be considered that the xenobiotic metabolism of the skin differs not only quantitatively but also qualitatively from that of the liver which is the central organ of xenobiotic metabolism [45]. Therefore, reasonable additional testing of their possible genotoxicity may be performed targeting cells of the skin. Such an evaluation may be performed *in vitro* using one of several skin cell culture models that have been developed, including the three dimensional reconstructed human skin model [46], as well as testing *in vivo* the possible genotoxic effect within the skin of animals treated topically with the test compound.

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74 Exogenous Ochronosis: Update

Cheryl Y. Levin and Howard I. Maibach

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74.1 INTRODUCTION

Ochronosis, characterized by a bluish-gray discoloration in connective tissue, exists in both endogenous and exogenous forms. The endogenous form, alkaptonuria, is an autosomal recessive disorder resulting from the absence of homogentisic acid oxidase (HGAO). HGA accumulates in the blood and binds to fibrillar collagen to induce skin coloration and joint arthropathy [1].

Exogenous ochronosis is clinically and histologically similar to its endogenous counterpart; however, it exhibits no systemic effects and is not an inherited disorder. It is characterized by an asymptomatic hyperpigmentation of the face, sides and back of the neck, back, and extensor surfaces of the extremities [34]. The associated ochronotic discoloration most commonly results from the use of hydroquinone, though resorcinol, phenol, mercury, picric acid, and antimalarials have been implicated as well [4,20]. Hyperpigmentation typically appears after 6 months of continual use of the product [44]. The highest reported incidence of this syndrome occurred in South African blacks [28]. In contrast, in United States, this condition is believed uncommon [9,30]. Interestingly, the European Union recently banned the use of hydroquinone in any products, because there is animal data to suggest that hydroquinone is fetotoxic and is capable of producing renal adenomas in rats [12,31]. The following reviews the clinical features and histological appearance, offending agents, and putative mechanisms of action associated with exogenous ochronosis. Some of the theories surrounding the high prevalence of exogenous ochronosis among South African blacks are discussed.

74.2 CLINICAL/HISTOLOGICAL FEATURES

The early reports of exogenous ochronosis described severe cases [18], though three stages have since been identified. Stage I involves erythema and mild pigmentation of the face and neck. Progression to hyperpigmentation with "caviar-like" papules occurs in stage II. The final stage includes papulonodules with or without surrounding inflammation [14].

Histologic examination of exogenous ochronotic lesions reveals yellowish-brown or green, curled banana-shaped ochronotic fibers [10]. In advanced stages, there is a degeneration of the ochronotic fibers and the formation of a colloid milium. Stage III presents with inflammatory mediators, including multinucleate giant cells, plasma cells, epithelioid cells, and histiocytes. Transepidermal elimination of pigment and pseudoepitheliomatous hyperplasia have also been described in severe cases [24,25].

Some biopsies exhibit histological similarity to sarcoidoisis, with a sarcoid-like granuloma surrounding the ochronotic material. The source of the sarcoid-like fibers is debated, with some authors suggesting that they represent abnormal elastic fibers [4,21] and others positing that collagen fibers form the ochronotic pigment [10,18,37,43].

74.3 SOUTH AFRICA VERSUS UNITED STATES

Several causative factors have been proposed to explain the remarkable discrepancy in incidence between the United States and South Africa. High concentrations of hydroquinone used in South African products prior to 1984 were linked with the increased incidence of exogenous ochronosis; South African skin-lightening agents contained 6–8% hydroquinone [6,17]. In contrast, as recommended by the FDA Miscellaneous Panel, less than 2% hydroquinone has been incorporated into U.S. products over the counter (OTC) in recent decades [33]. Irreversible depigmentation agents such as *t*-butyl alcohol and mercuric compounds were also incorporated into South African skin care until 1986. This may have additionally contributed to the increased exogenous ochronosis.

More than 15 years following the South African–mandated 2% limit on skin-lightening agents, exogenous ochronosis in

TABLE 74.1
Exogenous Ochronosis in the United States (Published Cases)

Number of Patients	Patient (Age/Race/Sex)	Putative Cause	Reference
1	58 B/F	2% HQ for 2.5 years and 5–6 times daily	[10]
2	75 B/F	2% HQ for 2 years	[21]
	49 B/F	OTC skin-lightening cream for 2 months	
1	Not stated	Not stated	[36]
1	72 B/F	1 month of darkening after using skin lightening creams since childhood	[9]
2	62 B/F	1% HQ for 2–3 years	[30]
	46 B/F	1% HQ duration unknown	
1	47 B/F	4% HQ for 18 months	[19]
3	Not stated	Not stated	[7]
1	36 Hisp/F	2% HQ for 4 months	[22]
1	53 B/F	2% HQ for 2–3 months	[13]
1	40 Cajun/M	Not stated	[11]
2	56 B/M	6.5–7.5% HQ for "many" years	[25]
	39 B/F	Skin-lightening cream for 5 years (unknown HQ concentration)	
2	44 B/F	2% HQ for 3–4 years	[32]
	56 B/F	OTC skin-lightening creams for 30 years	
1	72 C/M	Secondary to alkaptonuria which he has had for 37 years	[1]
1	59 B/F	2–4% HQ for many years	[41]
1	45 F	2% HQ used to treat melasma	[46]
	Race NG	EO associated with allergic hypersensitivity to HQ	
6	40–70 B/F	Not stated	[24]
1	50 Hisp/F	2% HQ for 30 years	[28]
2	47 B/F	Not stated. Heavy use of "bleaching creams" for months	[3]
	46 NA/M	HQ for 1 year. Percent HQ not stated	

South African blacks continues to assume epidemic proportions [14,16,18]. Following the South African ban on higher concentrations of hydroquinone, there was a marked growth of antiacne products containing resorcinol, a known ochronotic agent [27,42]. Hydroquinone and resorcinol are often simultaneously used in South Africa to achieve a more rapid lightening. Therefore, some theorize that the synergistic effect of the two products could help explain the increased exogenous ochronosis in South African blacks [6].

Additionally, in South Africa, a predominant formulation includes hydroquinone in a hydroalcoholic lotion. In fact, 46% of the South African market sold the alcoholic lotion OTC, while in the United States and other countries, these products are available by prescription only. The alcoholic vehicle significantly increases the skin penetration of hydroquinone and could therefore also be responsible for the continued high incidence of exogenous ochronosis in South Africa [5] (Table 74.1).

74.4 MECHANISMS OF ACTION

Hydroquinone, as a depigmenting agent, blocks the synthesis of melanin by inhibiting the tyrosinase enzyme. However, the etiology of hydroquinone-induced hyperpigmentation in exogenous ochronosis remains speculative. Some believe the ochronotic pigment represents abnormal collagen fibers, while others believe abnormal elastic fibers provide the pigment (Table 74.2).

TABLE 74.2
Proposed Etiologies of Hydroquinone-Associated Exogenous Ochronosis

- High concentrations of hydroquinone ACTIVATE tyrosinase, increasing melanin synthesis [8]
- Oxidized by-products of hydroquinone ACTIVATE tyrosinase [15]
- Hydroquinone is absorbed into the dermis after its effect on melanocytes has ceased, and is engulfed by fibroblasts that excrete the pigment [18]
- Melanocytic involvement [23]
- Sunlight activation of ochronotic pigment [33,35]
- Inhibition of local activation of homogentisic acid oxidase [36]

In a study by Chen and Chavin [8] on black goldfish skin, lower concentrations of hydroquinone inhibited tyrosinase, while higher concentrations had an opposite effect, activating tyrosinase, and thus putatively increased melanin synthesis. Engasser suggested that the oxidized by-products of hydroquinone may be responsible for the tyrosinase activation [15]. Since at least 30 different formulations of hydroquinone have caused exogenous ochronosis, others believe that the hyperpigmentation cannot result from the oxidized or contaminant breakdown products [45]. In a study by Hull and Procter [23], the melanocyte-free areas on the face of an ochronotic patient with vitiligo were not hyperpigmented, suggesting that melanocytes are involved in the development of ochronosis [44].

Others have proposed that topical application of hydroquinone inhibits the local activity of HGA, which then polymerizes to form ochronotic pigment [36]. Hydroquinone is a phenolic compound similar to HGA. In a study using beef liver in 1953, hydroquinone inhibited HGAO [39]. The proposed mechanism resembles that of endogenous ochronosis and is thus appealing; however, it does not address the prevalence of hydroquinone in the black population [13].

Since exogenous ochronosis is often limited to the face and neck, some suggest that sunlight is involved [33,35]. Findlay et al. [18] proposed the theory termed "melanocyte recovery," whereby after prolonged use of hydroquinone, melanocytes overcome the product's damaging effects and overreact to sunlight. Others contend that the hyperpigmentation represents regions in which more of the offending agent was applied [37].

74.5 TREATMENTS

Treatment of exogenous ochronosis remains difficult. Avoidance of offending agent(s) is beneficial, but it may require years before observing any improvement [18,21]. Tretinoin gel, trichloroacetic acid, and cryotherapy have been ineffective [13]. Retinoic acid improved some patients, but caused transient hyperpigmentation in others [38]. Result of treatment with sunscreens and low-potency corticoids has been variable as well. Dermabrasion successfully removed hyperpigmentation in one white female patient excluding the thin-skinned malar areas [29]. Diven et al. [13] utilized a combination of dermabrasion and CO_2 to successfully treat the periorbital and nasal thin-skinned regions of a black female patient. More recently, a Q-switched ruby laser and a Q-switched alexandrite laser were utilized to treat exogenous ochronosis with good results [3,28]. The efficacy of the pigment-specific lasers is thought to be secondary to disruption of the dermal ochronotic pigment fibers, and their subsequent removal through lymphatic drainage or transepidermal elimination [2,3]. While promising, these newer techniques must be tested in a controlled fashion with a larger population base to determine their true efficacy (Table 74.3).

TABLE 74.3
Efficacy of Therapies in Treating Exogenous Ochronosis

Therapy	Clinical Efficacy
Avoidance of offending agent	Beneficial, but slow improvement
Retinoic acid	Helpful for some patients; caused transient hyperpigmentation in others
Tretinoin gel	Ineffective
Sunscreen	Variable efficacy
Trichloroacetic acid	Ineffective
Low-potency corticosteroids	Variable efficacy
Dermabrasion	Beneficial, infrequently utilized
Dermabrasion and CO_2	Beneficial, infrequently utilized
Cryotherapy	Ineffective
Q-switched alexandrite laser	Beneficial, infrequently utilized
Q-switched ruby laser	Beneficial, infrequently utilized

Note: We await evidence-based studies assessing the efficacy of potential therapies in treating exogenous ochronosis.

74.6 CONCLUSION

The majority of exogenous ochronotic patients are black [3,13,24,25,29,35,41] though Hispanics [22,28], Native Americans [3], and Caucasians [29] have been reported as well. Exogenous ochronosis can be psychologically debilitating and, if misdiagnosed, patients may continue to apply more of the causative agent to lighten the affected areas, leading to further hyperpigmentation. This may result from the use of hydroquinone, although the concomitant ingestion of certain drugs may also cause an ochronotic-like hyperpigmentation [26]. The frequency and duration of application of an offending agent can affect the amount of drug absorbed into the skin. There has been citation of exogenous ochronosis occurring with lower concentrations of hydroquinone [9,30,40], though the incidence appears to be quite rare considering the great many units sold OTC for over 40 or more years. This is in direct contrast to the high incidence in South Africa and we await new insights about this.

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75 Diagnostic Patch Test: Science and Art

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75.1 INTRODUCTION

Patch testing constitutes the most important diagnostic and investigative method currently available for studying allergic contact dermatitis (ACD) in clinical practice. The procedure involves the epicutaneous application of a specific substance (allergen) that should induce a cutaneous inflammatory reaction in the susceptible (sensitized) person, while causing no reaction in a nonsensitized person. The local reaction, reproducing the dermatitis "in miniature," provides a visible representation of the subject's general ability to react to the substance. Therefore, patch testing employs the agent that causes the disease; it applies that agent to the target organ, and it reproduces locally the pathogenic and immunologic mechanisms and morphological changes of the disease itself. However, as a bioassay, patch testing still confronts several inherent methodological problems and requires strict observation of the technical aspects and critical assessment of the results. The issue of whether a positive patch-test reaction is causally linked to the disease being studied involves several pitfalls including the inherent risk of false-positive responses and the difficulties in assessing clinical relevance. Besides, the magnitude of the problem of false-negative results is largely unknown. These issues are scarcely mentioned in the literature and frequently overlooked in clinical studies on series of ACD patients. Recognizing the benefits of patch testing as well as all its possible pitfalls is of practical importance to the physician using this method for clinical diagnosis.

The criteria used to assess the efficacy of patch tests are similar to all diagnostic tests. We can consider general criteria, which are the same as those used to assess that of the therapeutic interventions; namely, effectiveness, safety, acceptability, and costs. There are also criteria specific to the assessment of tests, i.e., sensitivity, specificity, the relationship between sensitivity and specificity, reproducibility, predictive value, and likelihood ratio. These basic biostatistical concepts must be taken into consideration when validating a test as a diagnostic tool. The ultimate criterion for the usefulness of a diagnostic test is whether it supplements information beyond that otherwise available, and whether this

information leads to a change in management that finally has a positive impact on the patient's outcome.

75.2 WHY AND WHEN PATCH TESTING?

The major indication for patch testing is the investigation of a probable ACD. Patch testing constitutes, together with a detailed clinical history and a complete physical examination, a crucial step in the diagnostic workup. The diagnosis of ACD involves (1) demonstrating the existence of delayed hypersensitivity to one or several allergens, (2) demonstrating that the patient is exposed to the allergen(s), and (3) establishing that the hypersensitivity and the exposure explain the dermatitis under investigation.[1] In this sense, a positive patch-test reaction establishes that the subject has been previously exposed and sensitized to the allergen; nevertheless, this does not imply that the clinical exposure to the tested substance is the cause of the current dermatitis. The clinical history and additional provocative testing should determine whether there is a causal relationship between the attributable exposure and the clinical course of the dermatitis.[2] Although patch testing is primarily conducted according to the clinical history and physical examination, the diagnostic process is bidirectional and test results will guide further questioning and investigation. Reconsidering the history in the light of the test results can lead to the recognition of many concealed sources of causative exposure. Moreover, patch testing yields additional information that cannot be disclosed from the clinical history.

It has been stated that when predicting the causative allergen from clinical information, an experienced physician may be right in about 50% of the time, mostly when common allergens are involved. For less common allergens in the standard series, this conjecture will be correct in just about 10% of cases.[3] Few studies have assessed the value of the clinical history and examination in the prediction of the test results and the incidence and relevance of clinically unsuspected positive patch tests.[4–7] Cronin[5] studied 1000 patients by thorough clinical investigation and patch testing, and demonstrated that the accuracy of the clinical prediction varies depending on the characteristics of the clinical dermatitis and the causative allergen. In a small group of patients having contact sensitization as the exclusive cause of their eczema (7% of the total), the clinical anticipation of the patch-test results was good (70%). On the contrary, when the contact sensitization was incidental to the patient's primary dermatitis, the accuracy of clinical prediction was poor. Nickel was the most frequent sensitizer in women and the easiest to diagnose. Of the 84 nickel-sensitive women, the allergy was anticipated in 54 of them (64%). Chromate, the commonest sensitizer in men, was suspected only in 40% of the cases (19 out of 48). Sensitization to other common allergens such as lanolin and neomycin was predicted in 7 out of 44 patients (16%) and in 4 out of 53 (8%), respectively. Patch testing has been shown to be significant both in confirming contact sensitivities suspected from the clinical history and in unveiling unsuspected sensitivities. Podmore et al.[7] patch tested 100 consecutive patients, 41 of them were tested for screening purposes, e.g., eczema without an obvious allergic contact factor or clinical contact dermatitis without an obvious allergen. In 59 patients, a contact allergen was strongly suspected. Diagnosis was confirmed in 32 patients. In addition, 17 patients had 23 unexpected positive reactions. At least 50% of the unexpected reactions were considered relevant to the patient's skin condition. If only the clinically suspected substances are tested, then all other possible sensitivities that are not immediately evident from the history would be neglected. For this reason, a standard panel should be applied in all patients with suspected contact dermatitis.[4,8]

Patch testing should also be used to uncover contact allergy as a superimposed or complicating factor in endogenous or exogenous dermatitis other than ACD. Ideally, all patients with chronic or nonresponsive eczematous dermatitis should be considered for patch testing, specially those with hand or hand and foot dermatitis (irritant, dyshidrotic, hyperkeratotic, and even psoriasis and pustulosis palmaris et plantaris), stasis dermatitis, atopic dermatitis, and numular eczema.[9,10] Other patients in whom patch testing may be considered are those with unclassified eczema, eczematous psoriasis, essential pruritus, otitis externa, and suspected drug eruptions.[11–14] When these patients are assessed clinically but without patch testing, they may not be suspected of having an allergic component. Moreover, in many cases, the offending agent is present in the topical products prescribed, or self-administered, for the treatment of the primary disease. However, when testing these patients, bear in mind that, as the prevalence of ACD is deemed to be lower than in patients meeting the case definition of ACD,[1] the predictive value of patch testing will decline (see The Validity of Diagnostic Patch Tests).

Even when uncovering unsuspected contact allergies are important, the significance of these sensitivities to the clinical dermatitis varies substantially. Many times, no clinical relevance is found, while other times, avoiding exposure to the sensitizer may constitute the only measure that can be adopted to control the flares in a chronic, recalcitrant dermatitis.[13] The correct diagnosis and characterization of the causative agent(s) of the patient's dermatitis constitute essential prerequisites for adequate therapeutic and preventive measures to be established.[15–17] Conversely, without the use of diagnostic patch testing, the patient may be given needless prohibitions in a vain attempt to improve the dermatitis.

75.3 THE VALIDITY OF DIAGNOSTIC PATCH TESTS

75.3.1 Sensitivity, Specificity, and Predictive Value

The statistical principles that underlie the evaluation of diagnostic tests are frequently overlooked by clinicians. These principles are substantial in recognizing the inherent limitations that are present when applying diagnostic tests. The classic indicators for evaluating the validity of diagnostic tests are *sensitivity*, *specificity*, relationship between sensitivity and specificity, and *predictive value*. These indicators

Patch-test result	*Contact allergy*		Predictive value
	Present	**Absent**	
Positive	True positive (TP)	False positive (FP)	Positive (TP/TP + FP)
Negative	False negative (FN)	True negative (TN)	Negative (TN/TN + FN)
	Sensitivity (TP/TP + FN)	**Specificity (TN/TN + FP)**	

FIGURE 75.1 Sensitivity, specificity, and predictive value of patch-test results.

compare the diagnostic discrimination of the test to the reference criterion or gold standard, which, by definition, has a sensitivity and a specificity of 100%. The concept can be delineated using a 2×2 contingency table that takes into account the test result (i.e., interpreted as positive or negative) and the presence or absence of the disease being studied (Figure 75.1). The key to patch testing is to allocate the tested individuals either into those who are allergic to the test chemical and should have a positive result, or those who are not allergic and should have a negative result. Those instances in which the test result is positive, but no disease is present are called false-positive results. The negative test results found when disease was actually present are called false-negative results. The proportion of subjects with a positive test result out of all those with disease is known as the sensitivity of the test. In our scenario, it measures the proportion of allergic individuals that are correctly identified by the test; *ergo*, it measures how sensitive the test is to detect contact allergy. Specificity is the proportion of subjects without disease with an appropriate negative test result. It measures the proportion of individuals without contact allergy who are correctly identified by the test as nonallergic. In other words, sensitivity and specificity indicate the proportion of individuals who have been correctly identified as allergic or not allergic. These indices provide stable estimates of the test's diagnostic discrimination and can be applied to any diagnostic test irrespective of the characteristics of the population on which the test is used.[18–21] We can also determine in how many instances the test result is true out of all the test results that are positive (i.e., true-positive result). The percentage of true-positive results out of all the positive test results is referred to as the positive predictive value of the test (Figure 75.1). It represents the probability that a patient with a positive test result actually has the disease. Similarly, the percentage of true-negative results out of all the negative test results is referred to as the negative predictive value of the test. The positive and negative predictive values are of great importance for clinicians, who interpret the test results in a case-by-case basis. However, these values will vary depending on the prevalence of the disease in the population upon which the test is applied. Thus, the significance of a test result is determined not only by the sensitivity and the specificity of the test itself, but also by the prevalence of the condition in the studied population. If the rate of contact allergy in the population tested is low, then the negative predictive value increases and the positive predictive value decreases. Conversely, when the rate of allergic persons tested increases; i.e., patch testing is used mostly to confirm the clinical diagnosis; then the positive predictive value will increase at the same test sensitivity, while the negative predictive value will decrease.[18–22] These statistical considerations have decisive clinical implications. In clinical testing, positive reactions are at least 10 times less frequent than negative ones. Therefore, even assuming that the test has high specificity, false-positive reactions will have great impact on the proportion of true positives out of all positives elicited (i.e., the positive predictive value of the test). This substantiates the importance of achieving a high prevalence rate of truly sensitized patients through a careful clinical assessment before patch testing.[21]

The validity of a test is its intrinsic ability to detect which individuals have the target disease and which do not; therefore, it is based exclusively on the inherent sensitivity and specificity of the test. To validate a test as a diagnostic tool, we should be able to discriminate how many times the test has accurately classified the tested subjects. Achieving this goal requires knowing in advance which subjects had the disease being studied based on some reference test. The optimal design for assessing the accuracy of a diagnostic test is considered to be a prospective blind comparison of the test, and a reference test or gold standard in a consecutive series of patients from a relevant clinical population.[22] As patch testing constitutes the only reliable and readily available test for diagnosis of contact allergy, the gold standard for comparison must be a confident clinical diagnosis made through the exhaustive study of each case and fulfillment of a precise case definition, in terms of the clinical findings, history of exposure to the tested substance, and reproducibility of the response with an appropriate time course after exposure.[1] Alternatively, a repeated open application test (ROAT) or a controlled exposure to the tested substance can be envisaged as a reference for comparison. However,

these tests also have a certain degree of ambiguity and need further standardization.[23] Available data concerning validity of patch testing as a diagnostic tool are quite limited because, in clinical grounds, we usually do not apply diagnostic tests to groups of subjects who are known to have the disease we are trying to diagnose (i.e., with incontrovertible contact sensitivity to the substances being tested). Similarly, data regarding testing in subjects without contact dermatitis are scarce. To assess the validity of patch-test screening trays in the evaluation of patients with ACD, Nethercott and Holness[24] tested 1032 patients, 639 of them with the International Contact Dermatitis Research Group (ICDRG) standard series and 393 with the North American Contact Dermatitis Group (NACDG) standard series, with the use of Al-Test patches or Finn Chambers. They found that sensitivity, specificity, positive accuracy, negative accuracy, and validity index for the ICDRG and NACDG screening series were 0.68, 0.77, 0.66, 0.79, 0.72 and 0.77, 0.71, 0.66, 0.79, 0.74, respectively. Therefore, although both screening series scored relatively high, nearly 30% of all patch-test results were considered inaccurate. Note, however, that the authors considered those patients with positive test results in whom the investigation did not provide evidence to support clinical relevance (either present or past) as having false-positive tests. Similarly, patients with negative tests results to the screening series, in whom further testing revealed positive responses to other allergens, were taken to have false-negative screening tests.

The issue of patch-test validity is problematic in that patch testing does not represent a particular test such as serum glucose, but rather a technique of testing. Thus, sensitivity, specificity, and predictive values will vary depending on the allergens tested.

75.3.2 Reproducibility of Patch-Test Results

The value of any test depends on its ability to yield the same result when reapplied to stable patients. Reproducibility of patch testing, defined as the test's ability to give consistent results when testing is repeatedly performed on the same individual, has been frequently questioned. Some authors have pointed out the low reproducibility of the patch-test responses when testing was performed in duplicate on different body areas, such as the right and left sides of the back.[25,26] Gollhausen et al.[25] double-tested concomitantly on the left and right sides of the upper back in 35 patients with allergens from the standard series and some vehicles (ointments), and found that 43.8% of the positive allergic reactions were nonreproducible. Subsequently, they reported a higher incidence of nonreproducibility of duplicate patch testing using allergens in petrolatum and Finn Chambers (37.9%) when compared with TRUE Test™ (17.9%).[26] In a multicenter study from the German Contact Dermatitis Research Group,[27] 1285 patients were double-tested concomitantly with 10 allergens from the standard series and manually loaded patch-test systems. Nonreproducible allergic reactions were seen in 194 patients (15.1%). The authors concluded that the nonreproducible results were highly dependent on the allergen tested. In contrast with these results, other authors[28–32] have reported that the reproducibility of patch testing was high. Lindelöf,[28] while testing 220 consecutive patients, obtained a nonreproducibility rate of 9.5%, Bousema[29] obtained 93% of concordant allergic results, and Bourke et al.[30] reported 8% of completely discordant results and observed that many nonreproducible results were not relevant. Using ready-to-apply systems, the reproducibility rate was even higher. Lachapelle[31] tested 100 consecutive patients using Epiquick™ and observed a nonreproducibility rate of 4.2%, and Ale and Maibach[32] obtained 95% of concordant allergic results in 500 consecutive patients using TRUE Test. Therefore, differences in the reproducibility of patch testing are possibly mostly due to methodological aspects[32–34] (see 75.4 Problems Associated with the Patch-Test Methodology).

75.4 PROBLEMS ASSOCIATED WITH THE PATCH-TEST METHODOLOGY

Standardization of the patch-test technique is essential for reproducible results. Much work has been done to optimize and standardize patch-test materials and methodology. Yet, systematic studies for several important aspects are lacking. Several factors may influence patch-test results and many sources of unreliability still exist (Table 75.1).

The use of an appropriate vehicle is crucial. Vehicles influence bioavailability and subsequent percutaneous penetration of allergens.[35–38] Petrolatum remains the standard vehicle for most allergens, with the exception of the TRUE

TABLE 75.1
Sources of Unreliability in Diagnostic Patch Testing

Materials
Type of patch-test system
Different sources of patch-test allergens
Different vehicles and concentrations for some allergens
Uneven distribution of allergens in the vehicle
Methodology
Amount of allergen applied
Regional variations in skin absorption and responsiveness
Dissimilar pressure supported by the system according to the area of application
Criteria of patient's selection
Application and reading times
Interpretation of the responses (intraindividual and interindividual variability)
Technical
Partial or complete detachment of patches
Errors in the sequence of consecutive allergens
Biological
Unresponsiveness (overlooked intercurrent factors such as sun exposure and drugs), weak and doubtful responses
Summation of individual responses
Hyperresponsiveness and excited skin syndrome

Test. However, adequacy of petrolatum as a vehicle for many allergens has been questioned.[39–44] Patch-test suspensions in petrolatum contain undispersed allergen particles and both, the particle size and number, differ significantly between different test substances and different manufacturers.[39,40,42] This phenomenon was specially described for metal salt preparations.[40,41,43] The nonhomogeneous release of allergen from the vehicle may result in false-positive reactions.[43] Nowadays, efforts are being made to optimize the preparations and assure a homogeneous dispersion of the allergens.

Skin absorption can vary greatly depending on the patch-test system used.[45] Factors such as conformity to the skin surface and degree of occlusion could be responsible for differences on the kinetics of allergen penetration. Variations in the amount of material applied can lead to erroneous results.[33,46] Excessive amounts can provoke spill over and irritant reactions, while inadequate dosing may conversely result in false-negative and questionable reactions.[47] The amount of material applied with the Finn Chamber technique should approximate 15 μL (range 12–18 μL), but as a manually dispensed system, it varies within a much wider range.[46] Problems associated with application seems to be solved with the "ready-to-use" delivery systems, such as TRUE Test, which has been pharmaceutically optimized concerning stability, solubility, and bioavailability of the allergens. The allergen dosage has been determined by dose-response studies and the amount per unit area standardized.[48,49]

Further methodological sources of unreliability are the existence of regional and intraregional variations (e.g., upper versus lower back) of patch-test responses[50–52] and miscellaneous individual factors, namely, menstrual cycle,[53,54] and seasonal variations.[55] Finally, standardizing the application time is required to achieve comparable results.[56–62]

75.5 PROBLEMS ASSOCIATED WITH THE PATCH-TEST SUBSTANCES

75.5.1 Problems Associated with Allergen Characterization and Chemical Variety

Ideally, allergens should be chemically defined and have high purity and stability. Standardized, commercially available allergens should be used whenever possible.[3] Most allergens of the standard series are pure chemicals, such as nickel sulfate, cobalt chloride, formaldehyde, etc. Others are chemically defined mixes of allergens such as thiuram mix, mercapto mix, fragrance mix, or paraben mix. Neomycin sulfate consists of three different chemical substances—neomicyn A, neomicyn B, and neamine. Finally, some testing materials are complex natural products, such as balsam of Peru (CAS 8007-00-9; 8016-42-0), colophony, or wool alcohols. Much research is necessary to clarify the chemical structure of these natural materials and define and characterize their allergenic fractions.[63,64] Even for the well-defined allergens, the optimal form of presentation is debated. Nickel is a good example, for which the sulfate is the present standard. Some authors, though, consider that chloride is more adequate.[65] Gold and mercury present similar problems.[66,67]

75.5.2 Problems Associated with Allergen Mixes

Mixes are used as screening tests to depict contact allergy to one or more of its constituents. They were designed as a simple method of increasing the number of chemicals tested while decreasing the number of patches applied. Yet, the use of mixes involves problems of concentration, interference, stability, formulation, and validation.[68–84] To avoid the occurrence of irritant reactions, the allergens are incorporated at suboptimal concentrations, sometimes resulting in false-negative reactions.[68,70,76,77] The fragrance mix, introduced as a screening tool in the late 1970s following the work of Larsen,[73] contains eight fragrance materials: eugenol, isoeugenol, oak moss, geraniol, hydroxycitronellal, α-amylcinnamic aldehyde, cinnamic aldehyde, and cinnamic alcohol. It also contains the emulsifier sorbitan sesquioleate (SSO) at 5% concentration to achieve a satisfactory dispersion of the constituents in the petrolatum vehicle. It is considered that the fragrance mix detects 70–80% of cases of fragrance sensitization.[74,75] The originally used formulation containing 2% of each constituent ($8 \times 2\%$) frequently resulted in false-positive irritant reactions. Therefore, the concentration was lowered to $8 \times 1\%$. However, the currently used concentration causes false-negative reactions[76,77] and still induces irritant reactions.[78,81] There are also discrepancies between the patch-testing results with fragrance mix and its constituents. A positive reaction to one or more of the fragrance mix constituents is seen in only 40–70% of patients with a positive reaction to fragrance mix.[80–83] Possible explanations for this discrepancy have been proposed in the excellent review by de Groot and Frosch,[75] including

1. False-positive (irritant) reactions to the mix.
2. False-negative reactions to the constituents. This may be due to
   a. Cross-reactions between chemically related substances in the mix.
   b. An additive suprathreshold effect of allergens in the mix.
   c. Enhancement of the absorption of the mix constituents by the emulsifier SSO.
   d. Enhancement of the absorption of other constituents by a marginally irritant constituent of the mix.
3. Formation of a new allergen by two or more constituents of the mix ("compound allergy").[85]

Enders et al.[83] consider that most negative reactions to the individual constituents in patients with positive reactions to the mix are due to the emulsifier SSO, which optimizes the diagnostic power of the mix. Therefore, they recommended the addition of 1% SSO when testing with the individual ingredients of the fragrance mix. In a multicenter study of the EECDRG,[78] the value of adding SSO was also investigated by

testing patients with fragrance mix, its eight constituents with and without 1% SSO, and SSO itself. The test concentration for SSO was 20% in petrolatum according to previous studies.[86] Positive allergic reactions to SSO were observed in 0.7% of the patients. The authors recommended the addition of SSO to the standard series to adequately evaluating a positive reaction to the fragrance mix. Negative reactions to the mix with positive reactions to the ingredients also occur. De Groot et al.[76] tested 677 patients with fragrance mix ($8 \times 1\%$) and its eight constituents. Sixty-one patients (9%) reacted to the mix and to one or more of the ingredients, while four (0.6% of all patients and 6.2% of all fragrance-sensitive patients) reacted to one of the individual ingredients in the absence of a reaction to the mix, even upon retesting with serial dilutions, and therefore, were deemed to have false-negative reactions to the mix. Even if the proportion of false-negative results was low, given the high prevalence of fragrance allergy, the number of missed allergies with the currently used mix may attain clinical significance. Testing with the individual ingredients of the mix in those patients clinically suspected of having contact sensitivity, even when the reaction to the mix is negative, may contribute to solve this problem. The same consideration is valid when there is a suspicion of a false-positive reaction due to irritancy.[76] As discussed earlier, the currently used fragrance mix ($8 \times 1\%$ with 5% SSO) is not perfect. It causes both false-positive and false-negative reactions and leaves 20–30% of fragrance sensitivities undetected.

Geier and Gefeller[84] published a thorough study in 21,000 patients, concerning the rubber mixtures, from the database of the Informational Network of Dermatological Clinics (IVDK) in Germany. They focused their analysis on the reliability of patch testing with the mixes of rubber ingredients as a marker for the detection of contact allergy to any of its constituents. The gold standard for comparison was the breakdown patch testing and the sensitivity of the mix was defined as the proportion of patients showing positive results to the mix among the number reacting to any of its single constituents. Thiuram mix elicited positive reactions in 222 patients (9.8% of all tested patients). Of these, 162 (73%) reacted to 1 or more components and 60 (27%) did not react, hence, were considered as false positives. Of the patients negative to the mix, 32 (1.6%) reacted to 1 or more components, and were deemed to be false negatives. The biostatistics for thiuram mix were as follows: sensitivity, 0.84; specificity, 0.97; positive predictive value, 0.73; and negative predictive value, 0.98. For mercapto mix, the sensitivity was 0.57 and the specificity 0.99. The statistics for Paraphenylenediamine (PPD) black rubber mix were sensitivity 0.65 and specificity 0.99. Only 224 patients were tested with carba mix. Positive reactions were found in 20 patients (8.9%) and 12 of them were considered as false positives. In addition, 2 of the 202 negative reactions (1%) were considered false negatives. The sensitivity was 0.80 and the specificity was 0.94. The authors concluded that the sensitivity and specificity of thiuram mix were acceptable. However, in case of an allergic reaction to the thiuram mix, they recommended breakdown testing, as only about one-half of the patients positive to the mix had positive reactions to one of the individual components. Mercapto mix had a low sensitivity, consequently, its replacement by Mercaptobenzothiazole (MBT) was proposed. Similarly, they proposed the replacement of PPD black rubber mix by N-isopropyl-N′-phenylparaphenylenediamine (IPPD) mix. It was difficult to reach a conclusion for carba mix because of the small number of tested patients.

75.6 PATCH-TEST CONCENTRATION

The outcome of an individual patch test not only depends on the existence of delayed hypersensitivity to the tested substance but also on the test concentration and the delivered dose, which will depend on the amount of percutaneous absorption induced by the method of exposure.[45] Delayed sensitivity is a dose-related phenomenon and there is a threshold surface concentration of allergen required to induce sensitization or elicitation of the response.[87–99] Therefore, the concentration of the allergen has an essential role in determining the amount of positive test results to be obtained. The dose of allergen should be kept sufficiently high to detect allergy in weakly sensitized individuals but low enough to minimize irritant reactions and the risk of sensitizing the patient. Almost any substance is capable of inducing irritant responses depending on the concentration and the method of exposure. When a test substance has low irritant properties, it is possible to use a relatively high elicitation threshold concentration; hence, allergic reactions will more likely be elicited. Conversely, if the substance has a fairly high irritancy potential, then a lower elicitation threshold concentration will have to be used to avoid the induction of false-positive irritant reactions. In the latter circumstance, allergic reactions are less likely elicited, especially in weakly sensitized persons. Variations in the cutoff concentrations will determine changes in the balance between positive and negative results.[91–98] If the elicitation threshold concentration is raised, both true-positive and false-positive test results will increase and the number of false negatives will decrease; the sensitivity increases and specificity decreases. Conversely, if the elicitation threshold concentration is reduced, we will have less false-positive test results, but also more false-negative responses. The specificity increases but sensitivity will decline. Therefore, the sensitivity and specificity of the test as well as the predictive values are related to the elicitation concentration (Table 75.2).

The choice of allergen dose is frequently a delicate compromise; it should maximize the possibilities of obtaining true-positive results, while minimizing the anticipated number of false-positive irritant results in nonallergic subjects. Commonly, patch-test concentrations for many allergens, even for allergens in the recommended standard screening trays, have been established testing groups of patients supposed to have ACD. In this context, a concentration is considered adequate when capable of eliciting a reasonable proportion of true-positive tests results (i.e., positive results which were accepted to be in association with contact allergy to the test substance based on clinical grounds), while eliciting a reasonably low proportion of irritant results according to

TABLE 75.2
Effect of Changes in the Elicitation Threshold Concentration

Elicitation Threshold Concentration ↑		*Elicitation Threshold Concentration* ↓	
⇓		⇓	
True positives ↑	False positives ↑	True positives ↓	False positives ↓
True negatives ↓	False negatives ↓	True negatives ↑	False negatives ↑
⇓		⇓	
Positive Predictive Value ↑		*Positive Predictive Value* ↓	
Negative Predictive Value ↓		*Negative Predictive Value* ↑	
SENSITIVITY ↑	*SPECIFICITY* ↓	*SENSITIVITY* ↓	*SPECIFICITY* ↑

morphological criteria. However, cutoff concentrations would be better estimated employing serial dilution test technique on patients proved to be sensitive to the tested substance through controlled exposure, and also, on nonsensitive controls. Using this technique, it would be possible to establish the concentrations eliciting strong, optimal, and minimal reactions. Thus, the mean, standard error, and ranges of reactivity for the different allergens can be calculated. Quantitative data about irritancy of the different substances can be obtained as well. This procedure has been used to standardize some patch-testing materials such as TRUE Test. The cutoff concentration for TRUE Test allergens was determined as the minimum concentration that caused a 2+ reaction in at least 90% of sensitive patients.[3,48,49] The comparative multicenter studies with TRUE Test and Finn chamber technique indicate proximity in limits of weak sensitization and irritancy with nickel, dichromate, cobalt, balsam of Peru, fragrance mix, carba mix, and thimerosal.[100–103]

75.7 PROBLEMS ASSOCIATED WITH PATIENT SELECTION

Before patch testing patients, we should consider critically all the information about clinical history and physical exam and generate precise pretest probabilities of meeting the case definition for ACD. An accurate clinical history and a proper physical examination are the best diagnostic tests we ever have.[104] As previously mentioned, to ensure a high positive predictive value when patch testing individual patients, it is crucial to maximize the proportion of patients who meet the case definition for ACD. In other words, the technique of patch testing is most effectively utilized as a confirming tool in those patients in whom a diagnosis of ACD was made based on strict clinical criteria. Performing patch test as a "last recourse" for managing refractory patients, who otherwise do not meet the clinical criteria for ACD, would not be expected to yield good results.[18–22] In addition, to determine sensitization rates in epidemiological studies, not only does the test system have to be well defined, but even more so, the test population. Sensitization rates in an insufficiently characterized test population can hardly reflect the number of clinically relevant sensitizations in the general population. Only data on clinically relevant sensitizations from a uniformly selected and well-characterized test population are suitable for making inferences. The pattern of allergic contact sensitization in a population is influenced by individual factors, such as sex, age, presence of atopy, presence of diseased skin, as well as factors related to exposure, including chemical structure of the allergen, concentration, climate, and industrialization.[105] An unequal frequency of positive patch tests is to be expected among groups of patients who differ with respect to individual variables. Christophersen et al.[106] evaluated the influence of individual factors on patch-test results from consecutive patients in seven centers in Denmark during a 6-month period. They concluded that the results could only be compared after stratification or multivariate analysis and proposed a logistic regression model for standardization of the presentation of patch-test results.

75.8 PROBLEMS ASSOCIATED WITH THE APPLICATION OF MULTIPLE PATCH TESTS

With the premise of increasing the sensitivity of the patch-test procedure and detecting as many clinically relevant allergic subjects as possible, it is common practice to employ arrays of many test substances grouped as tests series in the routine evaluation of patients with suspect ACD. If the cutoff concentration for each individual allergen in the series were settled at a 95% upper confidence limit, then, from a statistical viewpoint, each time we test 20 substances in a nonsensitized person, there would be a 100% chance of eliciting a false-positive result from one of the substances tested. If we set the upper confidence interval at 99%, i.e., assuming a false-positive response rate of 1% for each substance, we still have a 20% possibility of eliciting a false-positive result each time we test 20 substances.[19,21] As we consider the tray of substances as a single screening test rather than an assemblage of individual substances, we are dealing with a confidence interval of 80%, well below the conventional 95% confidence interval used in other diagnostic tests. If we wish to use a 95% confidence interval for patch-test screening and reduce the number of false-positive reactions, it would be necessary to lower the cutoff concentration of the individual test substances, which will simultaneously reduce the true-positive response rate.

Alternatively, we can consider reducing the number of test substances in the standard screening series to the indispensable minimum to diminish the risk of false-positive reactions. The above-mentioned concepts stress the significance of assessing the clinical relevance of the positive reactions. Critical revision of the clinical history and use of pertinent additional tests are needed to establish the validity of patch-test results.

Diepgen and Coenraads[21] delineated another problem associated with testing multiple substances. When estimating differences in sensitization rates between two groups of subjects (e.g., between males and females or between atopics and nonatopics), we frequently perform pairwise comparisons using chi-square tests, one for each allergen tested, setting a *p*-value of .05 as statistically significant. In this circumstance, and for a series of only 10 allergens, there is a random possibility of over 40% of finding, by chance, a statistically significant difference for at least one allergen between the two groups. Therefore, this procedure increases the probability of a false rejection of the null hypothesis, concluding that there is a difference in sensitization rate between the two groups, when there is in fact no difference.

75.9 VALIDITY OF TESTING WITH THE STANDARD SERIES

Most clinical cases of ACD are caused by a relatively small number of chemicals. Because of this, patients are tested with a group of 20–25 relevant chemicals grouped in a "standard series" as a primary screening procedure for the diagnosis of ACD. These series have been recommended by research groups (ICDRG, EECDRG, NACDG), with minor changes in different countries due to regional differences in exposure to sensitizing compounds. Their constitution is based on statistic of allergens and they are periodically revised to adapt to changes in exposure, introduction of new environmental allergens, and information regarding irritation, active sensitization, etc.[107] When testing with these substances, it is reasonable to assume that most of the obtained positive reactions can be ascribed to contact allergy. The usefulness of this screening series has been confirmed.[24,108–112] It is generally believed that the standard series alone detects 70–80% of all contact allergies,[108] but, in a multicenter study in 4824 consecutive patients from five European contact dermatitis departments, the sensitivities detected by the standard series alone ranged from 37–73% depending on the testing institution.[109] Patients without a positive patch test to the European standard series and positive reactions only to additional allergens varied in frequency from 5 to 23%. Sherertz and Swartz[110] found 36% of positive reactions occurred to allergens in the standard series exclusively, and overall, 76% reacted to one standard allergen. Cohen et al.[111] tested 732 consecutive patients with the NACDG standard 20 allergens, the NACDG extended series, and other allergens if estimated necessary. Of these, 363 patients (50%) had positive patch-test reactions. Only 23% of patients had positive reactions to the standard series of 20 allergens alone, 37% had a positive reaction to a standard series allergen and an additional supplementary allergen. Therefore, 60% of the patients had at least one positive standard reaction. Forty percent of patients had positive reactions only to allergens in the supplementary group. Of the total cohort of 363 reactors, 221 patients (61%) were considered to have clinically relevant positive patch-test reactions. Only patients with current clinical relevance were included in this group. Those patients reacting only to a standard series allergen had a high rate of clinical relevance (69%). However, they accounted for only 23% of all patients with positive reactions and 15.7% of patients with current clinically relevant reactions. The remaining 74% of clinically relevant reactions were detected only with the use of supplementary allergens alone or in conjunction with the standard series. In a multicenter study from the IVDK in Germany,[112] 4140 patients were tested with the German standard series. Contact sensitization was diagnosed in 47% of patients tested, varying from 30 to 64% in different centers. Forty percent of all patients proven to have contact allergy, were diagnosed using the standard series alone, while 20% of cases were diagnosed solely with additional allergens (occupation-specific or material brought in by the patient). Veien et al.[113] tested 6759 patients with the European standard series over a 5-year period. Additional allergens were tested in 1450 of these patients. Positive reactions to the allergens in the standard series were seen in 1941 patients, while 236 of the 1450 patients (16%) also tested with substances not included in the standard series had one or more positive reactions. Of the 1941 patients with positive patch tests, 1705 (88%) reacted only to substances in the standard series, 98 patients (5%) reacted both to substances in the standard series and to additional nonstandard substances, while 138 (7%) reacted only to nonstandard substances.

These variations may be attributed to differences in the population tested (i.e., differences in exposure and in the number of additional substances tested). The absolute frequency of contact allergy in any population will never be known, but the more substances and products tested, the greater will be the observed percentage of sensitization.

75.10 VALIDITY OF TESTING WITH NONSTANDARD ALLERGENS

Most patients should be tested to a standard series; additional series or individual allergens will be selected depending on the history and the distribution of the dermatitis, the occupation, and the geographic area. The characterization of a patch-test allergen requires an adequate knowledge of its sensitizing capacity, its occurrence in the environment, and if possible, the results of testing of a large number of subjects. Most allergens from the standard tray and the most commonly used "aimed" trays are chemically defined materials of high purity and a large amount of clinical data has been accumulated concerning patch-testing concentrations. When testing with these substances, it is reasonable to assume that most of the obtained positive reactions have significance and that investigation to assess relevance is warranted. In contrast, testing with nonstandard allergens, other than those of the

recommended aimed trays should be undertaken with caution. These may be chemically pure substances, but often they are compound products and may contain unknown components. Even some components can be irritants; such as the case for many industrial products. Specialized textbooks regarding test's concentration for many nonstandardized materials are currently available.[114] This information is of practical value as a starting point when testing with these materials. However, remember that for several of these substances there has been little research concerning test concentrations and suitable vehicles. When testing chemicals for which there are limited data, we face with the same problems regarding the sensitivity and specificity of the test; besides, we have to determine the appropriate strategy of testing and the valid test concentration, and we lack the information to categorically substantiate any conclusion. Therefore, patch testing with those materials may have little to contribute diagnostically, unless there is a definite clinical suggestion of their responsibility in the causation of the dermatitis. Finally, there are substances for which no information exists, except the chemical composition provided through the material safety data sheets for industrial products or the list of ingredients for household, cosmetic, and toiletry products (Table 75.3). Usually, these products are technical grade chemicals; therefore, it should always be considered that they may contain unknown components. Should any substance be considered potentially irritant, an open use test may be envisaged. It should be performed with diluted substances, whose concentration can be progressively increased as far as no response either allergic or irritant appears.[1] It is often helpful to patch test an uncommon substance at two or three 10-fold serial dilution, such as 1.0, 0.1, and 0.01%. This procedure will prevent seriously irritant reactions and may help to distinguish between irritant and allergic reactions. If an allergen is serially diluted, a gradual reduction in the intensity of the reaction usually occurs, while an irritant reaction will tend to disappear abruptly. A gradually declining reaction, still distinctly positive at 0.01% or even 0.001%, constitutes highly suggestive evidence of contact allergy to the tested substance. It has been the usual practice to determine the "safe" or "nonirritating" test concentration for new materials, by testing in control groups. Before any human test is performed, complete toxicological information of the material must be procured. In addition, the control subjects should be followed up for 1 month to rule out active sensitization. It is recommended to test at least 20 control subjects based on the premise that if none reacts at the selected concentration, then, the testing is above the 95% one-tailed confidence limit. Usually, the test is performed with several concentrations, selecting the highest nonirritant concentration as the elicitation (cutoff) concentration. The highest nonirritant concentration is then applied to the subject with suspect ACD to the substance. The development of a positive patch-test response with the morphological attributes of an allergic reaction will constitute evidence of contact sensitization due to the substance.

TABLE 75.3
Sources of Information in Exposure and Limitations of Different Sources

Product labeling
Labeling depends on regulatory policies that can vary in different countries.
Substances used in the manufacturing process are usually not included.
Substances added to raw materials are frequently not included.
Material safety data sheets (MSDS)
Accessibility of MSDS is not mandatory in some countries.
Sometimes information is incomplete or out of date.
Information from manufacturers or suppliers
Depends on the manufacturer's cooperation.
Sometimes information is inadequate, incomplete, or out of date.
Products' lists or databases
Sometimes information is incomplete or out of date.
Textbooks
Sometimes information is incomplete or out of date.
Chemical analysis of the Products
Difficult to accomplish in complex products.
Methodology is still not available or not validated for certain substances.
Time consuming.

75.11 PROBLEMS DERIVED FROM THE READING AND THE INTERPRETATION OF PATCH-TEST RESPONSES

Even when the proposals of the International Contact Dermatitis Research Group in 1970 concerning a uniform terminology for patch-test reading were generally accepted and represented a great advance,[115] reading of patch-test responses needs to be considered eminently subjective and constitute one of the limitations of the method.[116] Patch testing is a perceptual test, based on inspection and palpation of the test area. As any test that involves human perception and judgment, patch testing is bedeviled by variability of reporting on results. There are two forms of variability: (1) Intraobserver variability (i.e., the phenomenon in which the same observer classifies the same test result differently on two separate occasions) and (2) Interobserver variability (i.e., the phenomenon in which different observers classify the same test result differently). In epidemiological studies, this variability is recognized as an inevitable consequence of the use of perceptual tests.

The significant point in assessing a positive patch-test response is ascertaining whether it represents an allergic reaction or a false-positive reaction. Typical morphological features of an allergic test response are erythema, edema, papules, and vesicles (or bullae). At least an erythematous infiltration should be present for a reaction to be considered allergic, while reactions that show only erythema without infiltration, frequently correspond to irritant or nonspecific reactions.[115] Allergic patch-test reactions are traditionally scored in terms of intensity, and a grading scale from 1+ to 3+ is now generally accepted for ranking these allergic reactions. It should be emphasized that such grading is

of no consequence in terms of the disease state allocation; a strong response does not necessarily mean that the individual will be more affected in a practical sense with environmental exposure than an individual with a less-intense patch-test response. The only important consideration concerns as to whether there exists sufficient morphological features to classify a response as allergic or not. Even when all reading systems are based on the same morphological features, there remains some variation in the exact definition of the different grades of this scale between the different working groups. Such minor differences of categorization may determine variations in interpretation of the responses.[116] Bruze et al.[116] studied the accordance in patch-test readings and showed that there is good accordance among various readers, except with the NACDG system. The morphological feature that seemed most difficult to evaluate was the papule, so, perhaps it would be convenient not to demand the existence of this feature as essential for the categorization of a patch-test reaction as allergic.

The time of reading has been standardized[117] but is somewhat variable between different patch-test clinics. Usually, the first reading is performed at day two (48 h) after patch-test application, approximately 30 min after taking off the patches, and the second reading is performed at 72 or 96 h. A delayed reading (1 week) is also recommendable because certain allergens, e.g., neomycin, may determine late positive reactions.[117,118] Patch-test results should be read at least in two successive opportunities, without which, their accuracy is seriously impaired. A single reading on day 2 (48 h) may determine that approximately 30% of the contact allergies detected by the standard series are missed, as compared with the number of allergies found when the tests are read repeatedly up until 1 week from patch-test application.[117] In addition, multiple readings are crucial in distinguishing false-positive reactions. However, if only one reading is feasible, it should be performed on day 3 or 4.[117] With corticoids, a 7-day reading is highly recommended.[119]

75.11.1 Weak and Doubtful Reactions

Doubtful (?) and weak reactions require a cautious interpretation and a careful consideration of the clinical circumstance. When a weak reaction correlates with the clinical picture, it may be significant.[120] Because of biological or technical reasons, there might be a variation in the intensity of the test response to the same allergen from time to time. To establish or rule out contact allergy, merely repeating the patch test may be sufficient to demonstrate that a doubtful or weak reaction is not consistently obtainable and therefore, likely represents a false-positive reaction. If required, patch-test concentration may be raised and additional tests such as intradermal testing or provocative testing may be performed.[1,2]

75.11.2 False-Positive Reactions

The ideal patch test should indicate contact sensitization and produce no false-positive or false-negative reactions. The background of false-positive test reactions is usually irritancy. Although the recommended test concentration for the sensitizers in the standard series is the result of extensive international experience on testing, some of the concentrations (e.g., for chromate and formaldehyde) have been chosen too close to the irritancy threshold to diminish the risk of obtaining false-negative reactions. It has been claimed that false-positive irritant reactions do not represent a practical problem to the experienced physician. However, irritant reactions are often morphologically indistinguishable from allergic reactions.[121] Likewise, weak allergic reactions can also be clinically indistinguishable from false-positive allergic reactions. The distinction is not necessarily provided by conventional histology, nor yet appropriately resolved by specialized immunological[122–125] or bioengineering techniques.[126–129] Multiple positive patch-test reactions should arouse the physician suspicion to the excited skin syndrome.[130–132] The open use or provocative test may sometimes distinguish an allergic from an irritant response, because open testing is far less likely than closed testing to produce an irritant reaction. Ideally, the ambivalent patch test should be repeated, and possibly incorporate a dose-response assessment (serial dilution).[133]

75.11.3 False-Negative Reactions

The frequency of false-negative reactions is difficult to evaluate. Even with appropriate patch-test material there may be several reasons for false negativity, most often insufficient penetration of the allergen. Along with the intrinsic sensitizing properties of the substance, we consider concomitant exposure factors that might enhance the percutaneous penetration, i.e., irritation, occlusion, heat, and mechanical trauma.[134–137] These factors cannot be reproduced in patch testing. Skin hyporeactivity is a poorly investigated phenomenon that has to be considered as a possible cause of false-negative reactions.[138]

75.12 ASSESSMENT OF CLINICAL RELEVANCE

The fact that contact allergy to certain allergen(s) has been reliably demonstrated by careful patch testing does not prove that such allergen(s) is responsible for the patient's dermatitis. The clinician must determine whether or not the allergen is relevant to the dermatitis, either as a primary cause or as an aggravating factor. Assessing the relevance of a positive patch-test reaction is complex and involves many confounding factors. Little or no data on clinical relevance is provided in most clinical studies. Moreover, there is no consensus as to its definition, how it should be scored and assessed.[139] According to the ICDRG criteria,[115] we consider that a positive patch-test reaction is "relevant" if the allergen is traced. If the source of a positive patch test is not traced, we consider it as an "unexplained positive." We refer to as "current" or "present" relevance, if the positive patch test putatively explains the patient's present dermatitis. Likewise, when the positive patch test explains a past clinical disease, not directly related to the current symptoms, we refer to this as past relevance. However, recurrent but discontinuous contact with an allergen can occur in some patients, providing difficulty in

discriminating between current and past relevance. A modified relevance scoring system was proposed by Lachapelle[140] for categorizing present and past relevance of positive patch-test reactions: the system codifies relevance scores from 0 to 3, where 0 = not traced, 1 = doubtful, 2 = possible, and 3 = likely. Therefore, 16 combinations can be pondered for each individual case. The NACDG utilizes a similar scoring system using the terms: "relevance possible," "relevance probable," and "relevance definite."[141] If an allergen produces a patch-test reaction and the patient is exposed to circumstances in which skin contact with materials known to contain the allergen is likely to occur, relevance can be said to be possible. If the allergen can be identified in products to which the patients comes in contact then relevance is probable. If provocative use tests (PUT) or ROAT with a product to which the patient is customarily in contact produces a positive reaction, the concentration of the allergen in the product is sufficient to elicit the dermatitis and the relevance is certain. From a practical perspective, establishing that a positive reaction has past relevance or possible relevance does not direct the clinician to intervene directly for the very problem for which past testing was performed. Reporting data, not presently relevant, serves as an important epidemiological role and may be useful in preventing further outbreaks of ACD in a patient. Yet, it does not provide the information that it is essential in the management of the present problem for which the patient is being evaluated.

Absolute proof of relevance is often unattainable, as it is frequently not known whether suspected products actually contain the implicated allergen in sufficient amount to elicit the dermatitis. Many times a positive reaction is judged nonrelevant because of insufficient environmental information. Guidelines for assessment of relevance have been proposed[2] (Table 75.4). Assessment starts with a comprehensive clinical history and physical examination, and should be supplemented by a rigorous environmental evaluation investigating the existence of exposure to the putative agent, characteristics of this exposure, and possible concurrent factors. Relevance scores and accuracy of the assessment are significantly improved by a comprehensive knowledge of the patient's chemical environment. Sometimes it is difficult to substantiate the presence of the allergen in the patient's environment. This may be due to the complexity in detecting certain allergens or to insufficient knowledge about the composition of different products. As a consequence, the relevance scores for different allergens vary; the easier the identification of the source of an allergen, the higher the relevance scores.[2,140–142] Additional tests, such as tests with product's extracts, ROAT, and PUT, may prove valuable in establishing a definite causative relationship.[143]

An important effect seen in clinical practice is the differential verification of positive and negative results. As relevance is not assessed for negative reactions, we fail to identify the false-negative test results. Moreover, doubtful reactions may be clinically relevant according to undeniable clinical criteria or follow-up testing. It could be worthwhile to ascertain whether doubtful (?) or weak (+) patch-test reactions yield a significantly different relevance score than stronger and presumably more reliable positive patch-test reactions.

TABLE 75.4
Suggested Guidelines for the Assessment of Relevance

1. Reinterrogate the patient in light of the test results.
2. Perform a workplace visit.
3. Seek cross-reacting substances.
4. Consider concomitant and simultaneous sensitization.
5. Consider indirect, accidental, or seasonal contact.
6. Obtain information about environmental allergens from databases and textbooks.
7. Obtain information from the product's manufacturer.
8. Perform chemical analysis of products.
9. Perform additional tests with the allergen and the suspected products or the suspected products' extracts (e.g., patch test with serial dilutions ROAT and PUT).

75.13 PATCH TESTING IMPACT ON PATIENT OUTCOME

Whereas some studies indicate that the prognosis of ACD is poor;[144] if the responsible allergen is identified, patient education and allergen avoidance can lead to significant improvement in the dermatitis. Patients assessed clinically without patch testing may not be suspected of having an allergic component, or a wrong allergen may be presumed. Patch testing provides an accurate mean of diagnosis that allows the physician to identify a specific chemical responsible for the dermatitis and initiate appropriate management alleviating patients' suffering without losing valuable time.[145,146] Moreover, allergen identification is fundamentally important to prevent recurrence. However, evaluation of the overall benefits of patch testing remains controversial.[147] Lewis et al.[147] evaluated the value of patch testing from the patient's point of view, trying to establish whether patch testing influenced the clinical course of the dermatitis. A postal questionnaire was sent to 135 consecutive patients 2–3 months after patch testing. Of the 105 patients (77.8%) who replied, 42 (40%) felt that their skin condition had improved since patch testing, 8 felt that their skin condition had deteriorated, and 55 did not think that their condition had changed. Of these 105 patients, 56 had 1 or more positive allergic reactions, which were considered relevant in 43 patients. Of the 43 patients with a final clinical diagnosis of ACD, 31 (72%) believed that patch testing was beneficial, while only 20 (32%) of 62 patients who had a final diagnosis other than ACD considered patch testing as being helpful. Even if patch testing was demonstrated to be beneficial, the short timescale of the study did not allow an assessment of its effects on long-term prognosis. Rajagopalan et al.[148] evaluated retrospectively the value of patch testing in 260 ACD patients using a standardized questionnaire and revising medical records.

In this preliminary evaluation, they concluded that when patch testing is performed on patients with a prediagnosis duration of 2 months to 1 year, the postdiagnosis duration of the dermatitis was considerably decreased when comparing with the nontested patients. They suggested that patch testing should not be delayed to the extent that prognosis becomes worse in terms of chronicity of symptoms. In a subsequent multicenter observational prospective study,[149] the quality-of-life outcome between patch-tested and nonpatch-tested patients was assessed using a previously validated dermatology-specific quality-of-life (DSQL) instrument. Five hundred sixty seven patients from 10 centers were studied. Centers were selected to obtain a mix of stratified degrees of usage of patch testing. Data on 431 patients at the 6-month follow-up showed a significantly higher improvement in each of the DSQL domains in patch-tested patients (43% of the total group) compared with patch-tested subjects. In addition, a significant difference was observed in the time required to confirm the diagnosis and the proportion of subjects with confirmed diagnosis between the patch-tested and the nonpatch-tested groups.

75.14 CONCLUDING REMARKS

The outlined evidence suggest that, even if patch testing has limitations from the standpoint of its validity, it can be effectively used as a diagnostic test to establish the presence of contact sensitization to the test chemicals. The allergens in the standard series recommended by the ICDRG, EEGDRG, and NACDG are most likely to disclose valid and reliable results. However, several aspects of the patch-test procedure should be considered to reduce the risk of spurious false-positive or negative responses. The strategy for maximizing the efficacy and accuracy of patch testing includes the adoption of strict criteria for the selection of patients, further standardization of the patch technique, improved use of dose-response assessments, and above all, refined and rigorous procedures for the assessment of clinical relevance of the patch-test reactions. Patients may suffer major changes in their lifestyle on the basis of patch-testing results; therefore, it is crucial to establish that the positive reaction is actually linked to the clinical dermatitis. Patch testing may furnish information that cannot be disclosed from the clinical history and usually proves essential to the adequate treatment and prevention of recurrence. Providing an objective proof of the allergic condition, patch testing is essential for patient cooperation in allergen avoidance. Recent decades have provided many advances, much remains to be done.

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76 Irritant and Allergic Contact Dermatitis Treatment

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76.1 INTRODUCTION

Utilizing barrier creams (BC) as well as wearing appropriate gloves and clothing is an important measure in industry to prevent or reduce irritant contact dermatitis (ICD) and allergic contact dermatitis (ACD) (Zhai and Maibach, 2004a; Wahlberg and Maibach, 2005). However, once ICD or ACD occurs, topical corticoids and other therapy approaches are "standard" therapy (Maibach and Surber, 1992; Weltfriend et al., 2004). This chapter critically reviews this topic.

76.2 AVOIDANCE

76.2.1 MOISTURIZERS

Moisturizers are frequently used to improve "dry" skin, and daily use may modify the skin surface's physical and chemical nature, so as to smooth, soften, and make more pliable (Zhai and Maibach, 1998; Kligman, 2000).

Moisturizers often contain humectants of low molecular weight and lipids. They are absorbed into the stratum corneum and there, by attracting water, increase hydration

Modified from Zhai, H., Anigbogu, A., and Maibach, H.I. Treatment of irritant and allergic contact dermatitis, in L. Kanerva, P. Elosner, J.E. Wahlberg, and H.I. Maibach (eds.), *Handbook of Occupational Dermatology,* Springer, Berlin, 2000, 402–411. With permission.

(Loden, 1997). Lipids, for instance, petrolatum, beeswax, lanolin, and various oils in moisturizers, are incorporated into formulations on the basis of their technical and sensory properties rather than on their possible epidermal impact (Loden, 1995, 1997). They may also penetrate the living epidermis, be metabolized, and significantly modify endogenous epidermal lipids (Wertz and Downing, 1990). A single application of a moisturizer did not cause long-lasting effects expressed as skin capacitance and conductance (Blichmann et al., 1989; Loden and Lindberg, 1991), whereas repeated applications of a moisturizer twice daily for 1 week produced a significant increase in the skin conductance for at least 1-week posttreatment (Serup et al., 1989).

Urea, a physiological nonallergenic substance (Serup, 1992a; Swanbeck, 1992), can decrease reversibly the turnover of epidermal cells (Hannuksela, 1996), and may also enhance the penetration of other substances into skin (Feldmann and Maibach, 1974; Wohlrab, 1990; Serup, 1992a). Other effects include binding water in the horny layer, antipruritic, and reducing irritant dermatitis (Serup, 1992a,b; Swanbeck, 1992; Loden, 1996).

Zhai and Maibach (1998) reviewed the subject of moisturizers in preventing ICD. Extensive data on the physiology, pharmacology, and toxicology of moisturizers can be found in Loden and Maibach (2005).

76.2.2 Barrier Creams

BC are designed to prevent or reduce the penetration and absorption of hazardous materials, preventing skin lesions or other toxic effects from dermal exposure (Orchard, 1984; Frosch et al., 1993a,b; Lachapelle, 1996; Zhai and Maibach, 1996a,b). Their efficacy has been investigated by *in vitro* and *in vivo* studies (Frosch et al., 1993a; Lachapelle, 1996; Wigger-Alberti and Elsner, 1998; Zhai and Maibach, 1996a, 2004b). However, their actual benefit remains subjudice in clinical trials (Frosch et al., 1993a,b,c,d; Goh, 1991a,b; Goh and Gan, 1994; Lachapelle, 1996; Treffel et al., 1994; Treffel and Gabard, 1996; Wigger-Alberti and Elsner, 1998). Inappropriate BC application may exacerbate rather than ameliorate (Goh, 1991a,b; Frosch et al., 1993a,b,c,d; Zhai and Maibach, 1996b). In practice, BC are usually recommended only for low-grade irritants (water, detergents, organic solvents, cutting oils) (Frosch et al., 1993d; Zhai and Maibach, 1996b; Wigger-Alberti and Elsner, 1998). BC are also used to protect the face and neck against chemical and resinous dust and vapors (Birmingham, 1969).

Reasons, mechanisms, application methods, and general topics of BC can be found in Zhai and Maibach (2004a), as well as related chapters of this book.

76.2.3 Protective Gloves and Clothing

Gloves may provide certain protective effects against corrosive agents (acids, alkalis, etc.) (Boman et al., 1982, 2005; McClain and Storrs, 1992; Wigger-Alberti and Elsner, 1998). Protective clothing as well as other personal devices also play a critical role (Mathias, 1990; Davidson, 1994). Note that protective clothing may trap moisture and occlude potentially damaging substances next to the skin for prolonged periods and increase the likelihood that dermatitis will develop (Mathias, 1990; Davidson, 1994). The first line of defense against hand dermatitis is to wear gloves, but in many professions it is impossible to wear gloves because of the loss of dexterity. In some instances, an alternative would be to utilize BC. Note that many gloves do not resist the penetration of low molecular weight chemicals. Some allergens are soluble in rubber gloves, and may penetrate the glove and produce severe dermatitis (Mathias, 1990; Estlander et al., 1996; Wigger-Alberti and Elsner, 1998; Menné and Maibach, 2000; Chowdhury and Maibach, 2005; Boman et al., 2005). Recently, allergy to rubber latex has become a growing problem (Estlander et al., 1996; Wigger-Alberti and Elsner, 1998; Menné and Maibach, 2000; Chowdhury and Maibach, 2005; Boman et al., 2005), and workers can develop contact urticaria syndrome including generalized urticaria, conjunctivitis, rhinitis, and asthma (Amin and Maibach, 1997; Wigger-Alberti and Elsner, 1998). Updated document details in this area can be found elsewhere (Chowdhury and Maibach, 2005; Boman et al., 2005).

76.3 TREATMENT

76.3.1 Corticoids

Hydrocortisone, which became available in the 1950s, was shown to be efficacious in eczematous dermatoses (Sulzberger and Witten, 1957). The next major advance in topical corticoid therapy came with the introduction of triamcinolone acetonide, followed shortly after by flucinonolone acetonide. The early 1970s saw the introduction of the 21-acetate derivatives of fluocinolone acetonide with more biological activity than others. Since the late 1970s many potent topically active glucocorticoids have been introduced, including desoximetasone, clobetasol propionate, and betamethasone-17-dipropionate.

76.3.2 Mechanism of Action

Corticoids being lipophilic in nature permeate the skin by passive diffusion.

Following the penetration of the cell membrane, corticoids bind with specific cytoplasmic receptors. These receptors have been demonstrated in all target tissues including the skin (Ballard et al., 1974).

Since inflammation is the endpoint of the immune response, the anti-inflammatory and immunosuppressive effects of corticoids may overlap (Blackwell et al., 1980; Haynes and Muraud, 1985; Hirata et al., 1980; Parrillo and Fauci, 1979; Thompson and Van Furth, 1970; Vernon-Roberts, 1969).

The mechanisms by which topical corticoids cause vasoconstriction remain unclear (Altura, 1966; Ginsburg and Duff, 1958; Juhlin and Michaelsson, 1969; Solomon et al., 1965).

The effects of topical application of corticoids on human mast cells have been examined (Lavker and Scheckter, 1985). Two potent corticoids, clobetasol-17-propionate and fluocinonide, produced greater than 85% decrease in histamine content over 6-week treatment. The first signs of cells containing sparse amounts of mast-cell granules were apparent

14-day poststeroid treatment. By 3 months, histamine levels returned to normal. This work suggested a possible treatment for one human mast-cell disease, urticaria pigmentosa, and a possible additional mechanism of action of corticoids. Maibach and Surber (1992) and Korting and Maibach (1993) provide additional details.

76.3.3 Percutaneous Penetration

Following topical application, corticoids penetrate the stratum corneum and are absorbed into the epidermis. The efficacy and toxicity are related to corticoid penetration. Corticoids may act on the epidermis, the dermis, or both.

Topical corticoids applied to diseased skin will be absorbed into the systemic circulation. When administration is chronic or when large areas of skin are involved, the absorption may be sufficient to cause systemic effects including cushinoid changes and adrenocortical suppression.

Topical corticoids are minimally absorbed from healthy skin. On the forearm, approximately 1% of the applied dose of hydrocortisone penetrates (Feldmann and Maibach, 1966). Other corticoids for which data exist are not necessarily absorbed to a greater degree than hydrocortisone (Feldmann and Maibach, 1968), suggesting that they may owe their increased efficacy to their potency rather than to enhanced penetration.

76.3.4 Clinical Formulations and Potency of Corticoids

Corticoids form a vast range of compounds and formulations with varying effects. Table 76.1 groups topical corticoids according to relative potency, largely based on the vasoconstrictor assay (Stoughton, 1972). The formulations in each group are only roughly equipotent. The greater the potency, the greater the therapeutic efficacy and likelihood, therefore, of more adverse effects.

Superpotent formulations include clobetasol propionate, optimized betamethasone diproprionate, and difluorosone, and must be used with caution; they have the potential for significant topical and systemic side effects far in excess of other currently utilized formulations.

76.3.5 Vehicles

The potency of topical corticoids can be further enhanced by enhancing percutaneous absorption. One such way of optimizing absorption is by altering the formulation vehicle (Stoughton, 1972). Ointment bases tend to give greater activity to the corticoid than do cream or lotion vehicles (Stoughton, 1972).

76.3.6 Adverse Effects

All absorbable corticoids possess the ability to produce adrenal suppression (Carr and Tarnowski, 1968; Scoggins and Kliman, 1965). The degree of suppression is related to potency. Fortunately, plasma cortisol usually returns to normal within 3 days when the superpotents are discontinued—at least in short-time application studies (Levin and Maibach, 2002).

TABLE 76.1
A Partial List of Topical Corticoids Available in the United States Ranked According to Their Potencies

Drug	Potency (%)
Lowest potency	
Hydrocortisone	0.25–2.5
Methylprednisolone acetate	0.25
Dexamethasone[a]	0.04
Dexamethasone[a]	0.1
Methylprednisolone acetate	1.0
Prednisolone	0.5
Betamethasone[a]	0.2
Low potency	
Fluocinolone acetonide[a]	0.01
Betamethasone valerate[a]	0.01
Flurometholone[a]	0.025
Aclometasone dipropionate	0.05
Triamcinolone acetonide[a]	0.025
Clocortolone pivalate[a]	0.1
Flumethasone pivalate[a]	0.03
Intermediate potency	
Hydrocortisone valerate	0.2
Mometasone furoate	0.1
Hydrocortisone butyrate	0.1
Betamethasone benzoate[a]	0.025
Flurandrenolide[a]	0.025
Betamethasone valerate[a]	0.1
Desonide	0.05
Halcinonide[a]	0.025
Desoximetasone[a]	0.05
Flurandrenolide[a]	0.05
Triamcinolone acetonide[a]	0.1
Fluocinolone acetonide[a]	0.025
High potency	
Betamethasone dipropionate[a]	0.05
Amcinonide[a]	0.1
Desoximetasone[a]	0.25
Triamcinolone acetonide[a]	0.5
Fluocinolone acetonide[a]	0.2
Diflorasone diacetate[a]	0.05
Halcinonide[a]	0.1
Fluocinonide[a]	0.05
Highest potency	
Betamethasone dipropionate[a] in optimized vehicle	0.05
Diflorasone diacetatea in optimized vehicle	0.05
Clobetasol propionate[a]	0.05

[a] Fluorinated steroids.

Source: Zhai, H., Anigbogu, A., and Maibach, H.I., in *Handbook of Occupational Dermatology*, Springer, Berlin, 2000, 402–411. With permission.

Certain factors increase the penetration and therefore the tendency to suppression; application to large surface areas; occlusion; inflammed skin; and higher concentrations. Of concern in children is growth retardation associated with excessive and prolonged use of topical corticoids (Bode, 1980; Munro, 1976; Vermeer and Heremans, 1974; Weston et al., 1980).

76.3.7 Dosage and Administration

Most physicians prescribe topical corticoids with little or no thought as the number of milligrams of material per surface area of skin. There is a dosage–response relationship, with increasing efficacy closely following increased dosage. It is therefore important to make an estimate of the quantity a patient would require in any given condition. Fortunately, most manufacturers provide a standard or regular concentration yielding the desired therapeutic result for most patients. For instance, triamcinolone acetonide is available in 0.025, 0.1, and 0.5% formulations. Many patients with corticoid-responsive dermatoses need only the 0.025% formulations.

The standard trade concentrations suffice for most patients. In the more resistant diseases, higher concentrations should be considered. For instance, approximately 1% of a 0.25% hydrocortisone solution is absorbed from the forearm. Increasing the amount applied per unit area of skin 10-fold, increases the amount absorbed four times (Feldmann and Maibach, 1967).

Regional differences in response are partially based on the differences in penetration of skin in various areas. Thus, areas with increased permeability, such as the scrotum, eyelids, ears, scalp, and face respond far better to topical corticoids than such areas as the dorsa of the hands, extensor surfaces of knees and elbows, and the palms and soles (Mckenzie and Stoughton, 1962).

76.3.8 Occlusion

Occlusion of 96 h with an impermeable film, such as plastic wrap, constitutes a most effective method of enhancing penetration, yielding approximately a 10-fold increase (Feldmann and Maibach, 1965). Specifically, with occlusion, penetration of hydrocortisone on the forearm increases from 1% of applied dose to 10%. There are, however, obvious problems associated with occlusion therapy—the plastics are sometimes uncomfortable, warm, and troublesome to use. Side effects encountered with occlusion include miliaria, bacterial, and candidal infection.

Occlusion has the added advantage of keeping the drug on the skin by preventing rubbing off onto clothing. We do not have data delineating the effect of duration of occlusion on percutaneous penetration with topical corticoids.

76.3.9 Frequency of Application

Previously, patients applied topical corticoids three to four times daily. Studies on the percutaneous absorption of hydrocortisone failed to reveal a significant increase in absorption applied on a repetitive basis compared to a single dose (Lagos and Maibach, 1998). Clinical trials of various corticoids suggest that less-frequent applications are equally effective (Fredrickson et al., 1980). In view of the relatively slow process of corticoid absorption, a phenomenon referred to as the "reservoir effect," (Vickers, 1963) there may not be any advantage in frequent applications.

Acute tolerance (tachyphylaxis) to vasoconstriction and antimitotic effects of and suppression of epidermal DNA synthesis by topical corticoids have been demonstrated (Du Vivier et al., 1982; Du Vivier and Stoughton, 1976). This suggests that the resistance clinically observed after prolonged use might be prevented by less intensive therapy, such as daily application with short resting periods between treatment courses (Barry and Woodford, 1977; Miller and Munro, 1980). Another study examining corticoid tachyphylaxis used fluocinolone acetonide under occlusion to the forearm and induced wheal and flare to histamine with the prick technique (Singh and Singh, 1986). By the eighth day, the wheal was nonexistent, adding now a third tachyphylaxis phenomenon.

76.3.10 Anatomic Variation

Large regional variations in percutaneous absorption of compounds are determined by factors including hair follicle density, thickness of the stratum corneum, and vasculature of the region (Cronin and Stoughton, 1962). This suggests that for areas of higher penetrability such as the face, scalp, scrotum, axilla, and the groin, smaller doses are required and occlusion is not needed (Feldmann and Maibach, 1967).

Little quantitative information is available on how much penetration is increased in diseased skin (Aalto-Korte and Turpeinen, 1995). In initial studies, it was noted that skin with only minimally involved atopic dermatitis allowed for a severalfold increase in penetration; psoriatic plaques had no significant increase, whereas exfoliative psoriatic skin had little barrier to penetration.

76.4 CONTROLLED TOPICAL EFFICACY STUDIES: IRRITANT AND ALLERGIC CONTACT DERMATITIS

76.4.1 Irritant Dermatitis

Most physicians employ topical corticoids in irritant dermatitis; however, several controlled studies in experimental irritant dermatitis to sodium lauryl sulfate show either no, or a negative (Van der Valk and Maibach, 1996), or a minimal effect (Ramsing and Agner, 1995).

76.4.2 Allergic Contact Dermatitis

Several studies document some degree of efficacy when high potency corticoids are applied after the acute phase (Funk and Maibach, 1994). Considering the massive amounts prescribed, the data are limited—possibly because this has long been the standard of care.

76.4.3 Immunosuppressives

Cyclosporin, tacrolimus, and azathioprine are used in unusual instances. See Menné and Maibach (2000) for details.

76.4.4 UV Light

Most patients with ICD and ACD may be controlled by topical therapy and protective measures. But, some cases cannot be

controlled either topically or by acceptable doses of systemic corticosteriods. In these situations, UV treatment should be considered. Christensen (1994) and Menné and Maibach (2000) provide the details for UV treatment regime.

76.4.5 Grenz Ray

Grenz ray may act as an adjunct topical therapy in some chronic cases. In addition, it is extremely suitable if one considers the sparing effect of Grenz radiation on hair roots, sebaceous, and sweat glands. Details are provided by Lindelöf (1994) and Menné and Maibach (2000).

76.5 CONCLUSION

Taken together, most patients respond to current therapy; yet, considering the frequency of these conditions, much evidence-based data and refinement await investigation.

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77 Factors Affecting Children's Susceptibility to Chemicals

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77.1 INTRODUCTION

Environmental health regulations aim to protect populations from exposure to toxic chemicals and other environmental agents that can be harmful to health. The risk of an adverse health effect depends on several factors including the agent's toxicity, the level and duration of exposure, and the organism's sensitivity. There is concern that children are often more sensitive than adults given comparable exposures: their likelihood of developing adverse effects may be higher, their response more severe, their symptoms different, or impacts on them may not be apparent until long after the exposure has occurred. In recent years, research has aimed at understanding child–adult differences in sensitivity and risk, prompted by efforts to ensure that regulations are adequately protective. While factors that contribute to these differences have been identified, many knowledge gaps still exist.

Children are distinct from adults in terms of biological and behavioral characteristics that can influence chemical toxicity. Furthermore, these characteristics are not uniform throughout the development. This implies that sensitivity or susceptibility to a toxic chemical may differ by age or by childhood stage. These differences and their effect on risk are not well understood, and relevant information is limited. However, potentially significant periods of higher sensitivity throughout development can be identified using available information on toxicity-relevant factors.

This chapter describes, by developmental stage, changes in biological factors that affect the activity of toxicants in the body, also noting behavioral changes that can influence exposure. It concludes by suggesting how these might influence susceptibility and sensitivity throughout development. Some key concepts are defined next in this chapter, while certain aspects of this issue—such as organ system development, overall growth, sex differences, and inter-individual variation among children—are beyond the scope of the discussion.

77.1.1 Susceptibility and Sensitivity

The terms *susceptibility* and *sensitivity* suggest that the impacts of an exposure may be greater for some populations or individuals. There is, however, a subtle distinction between them as often used in the context of environmental health, and as used in this chapter. Susceptibility refers to a capacity characterized by factors, biological or physiological, that can modify the effect of a specified exposure, potentially leading to higher health risk at a given exposure

level. Sensitivity refers to the capacity to experience higher risk due to the combined effect of differences in exposure and susceptibility.

77.1.2 Toxicokinetics

The activity patterns of a toxicant's absorption, distribution, metabolism, and elimination are collectively referred to as toxicokinetics. These processes delineate the pathway of a chemical from its uptake through to its removal from the body. Absorption occurs as chemicals enter the blood circulation, mainly through the gastrointestinal tract, the lungs, and the skin. Once in the bloodstream they are distributed to body tissues and organ sites, where they are available for diffusion or transport into cells. Biotransformation or metabolism is a process that changes the properties of a chemical to facilitate excretion and maintain homeostasis; it is catalyzed by enzymes in the liver and other tissues. Elimination occurs through the kidney by excretion in urine and feces, to a lesser extent through other body secretions, and for certain chemicals through the lung.

77.1.3 Child

The term *child* has various definitions across disciplines and depending on the context, but the underlying notion is that of a developing organism. Development spans the time period from conception to adulthood and is often divided into categories or stages. The following life stages are used consistently in this chapter: "Fetus" (conception up to birth), "Neonate" (birth up to 1 month), "Infant" (1 month up to 2 years), "Preschool" (2–6 years), "School" (6–12 years), and "Adolescent" (12–18 years). The lower bound of these categories is inclusive of the year or event that denotes it.

77.2 TOXICITY-RELATED FACTORS BY DEVELOPMENTAL STAGE

Several biological processes contribute to the absorption (gastrointestinal, pulmonary, or dermal), distribution, metabolism, and elimination of chemicals from the body. These develop at variable rates, reaching maturity at different points in time during childhood. Developmental stages are also associated with behaviors that can contribute to differential exposure or dose of environmental chemicals.

77.2.1 Prenatal Stage

The prenatal stage is characterized by actively developing processes of growth, cell differentiation, and migration. Fetal exposure to hazardous chemicals can have effects of varying severity, from overt abnormalities to subtle or latent health effects. The timing of an exposure influences the outcome considerably, as particular time periods are associated with different degrees of resilience or susceptibility.[1–3] Toxicity during the first week of gestation may result in pregnancy termination or, at the other extreme, apparently normal development, albeit with the possibility of not readily observable effects such as retarded development of blastocyst cells. From the second or third week onward, there is a wider range of possible outcomes and biological targets.

Factors that can affect risk include, in addition to timing of exposure, the toxicity of the chemical, the chemical concentration or dose, the exposure route, maternal disease, and the genotype of the mother or fetus.[3]

77.2.1.1 Fetoplacental Metabolism, Distribution, and Excretion

Chemical substances can enter the maternal circulation through exposures during pregnancy, as well as mobilization of substances previously stored in the mother's body.[4] The transfer of chemicals from the mother to the fetus takes place primarily by simple diffusion via the placenta, which also provides nutrition and enables functions such as respiratory gas exchange and excretion. The process of diffusion becomes increasingly efficient after the fourth week of gestation. It depends on the properties of the chemical, placental blood flow, pH gradients, and placental metabolism. Transfer is favored for substances that are small, not protein bound, non-ionized and lipid-soluble, while accumulation is more likely for weak bases.[2,5–7]

The fetal placenta has some metabolic capacity that can influence the toxicity of a small number of xenobiotics (e.g., aniline, aflatoxin B1, *p*-nitroanisole). Few cytochrome P450 (CYP450) isoforms with chemical metabolizing ability are present in the fetus, and the activity of phase I and II processes is immature.[2,7–13] In general, feto-placental metabolic reactions function at low rates (with the exception of sulfation), and their effect is secondary to that of maternal metabolism. This can be protective when metabolic activation is required for a chemical to become toxic. For example, studies have shown that acetaminophen overdoses during pregnancy can lead to maternal hepatotoxicity without liver cell injury for the fetus: lacking a well-developed CYP450 system, acetaminophen is not converted to its hepatotoxic metabolite.[4,14]

The level of binding proteins in the maternal and fetal circulation fluctuates but is generally low prior to the third trimester.[7,15] This may lead to relatively high free fractions of a chemical and greater equilibrium concentrations at target tissues, increasing the likelihood of target organ toxicity. Fetuses have a relatively high percentage of body water versus body fat. Thus, lipophilic chemicals are more likely to accumulate in the brain and other lipid-rich targets.[4,16] The brain also lacks protection from an immature blood–brain barrier, an interface that—when fully developed—limits passage of chemical substances.

The fetus can filter and excrete maternal plasma water into the amniotic cavity by the eighth week of gestation. Fetal urination is the major source of amniotic fluid, which recirculates through swallowing after the first trimester and as early as the 10th week of gestation.[13,17] This could prolong fetal exposure to certain water-soluble substances through the gastrointestinal and dermal routes.

77.2.1.2 Gastrointestinal, Dermal, and Pulmonary Function

The structure of the gastrointestinal tract begins to develop in early prenatal stages, with specialized features appearing mostly in the second and third trimesters.[18] Development of functional processes follows; some of these mature in early prenatal stages though others reach maturity at birth or in the first year of life.

The fetal dermal barrier function is immature since the stratum corneum is not completely formed for almost the entire prenatal stage. The genesis of this outer-most layer of the skin does not begin until the final quarter of gestation, and is completed just before birth.[19,20] Dermal permeability decreases as gestational age increases, up to 35 weeks before birth.[21]

The formation of the lung begins around the 26th day of gestation, and its maturation follows several prenatal and postnatal stages of structural and functional developments.[22,23] Fetal ventilatory activity occurs irregularly and without gas exchange (the lung is filled with fluid), but has a role in preparing the fetus for extra-uterine breathing.[24] Alveolarization is thought to begin shortly before birth but the larger part of it occurs postnatally.

77.2.2 Postnatal Stages

77.2.2.1 Exposure Considerations

Newborns are dependent on their mother and other adult caregivers for nourishment, mobility, and protection from hazards in their environment. Exposures may be prolonged if adults do not take protective actions, such as withdrawal from sources of contaminants. Neonates may come into contact with chemicals in the environment through ingestion of breast milk or formula and water, through inhalation, and through dermal contact.

77.2.2.1.1 Diet, Behavior, and Activity

Growth and development during infancy, particularly in the first year of life, bring changes in physiology, intellect, and behavior. Infants begin to lose primitive reflexes and become more interactive with their environment around 3 or 4 months after birth. As head control and visual fields improve, they become capable of grasping objects and exploring them with their mouth. Object-to-mouth and hand-to-mouth activity are associated with nondietary oral intake, a significant exposure route during this life stage.[25] These activities can lead to ingestion of chemicals that accumulate on surfaces at lower elevations where infants spend much of their time. Studies of exposure to lead in soil, paint, or house dust, as well as chlorpyrifos on toys and other surfaces indicate the importance of this pathway.[26,27] Frequent mouthing behavior can contribute substantially to the ingested dose of chemicals especially if present at relatively high levels. Additional capabilities emerging during infancy, such as head control, sitting, finger feeding, and use of utensils, facilitate changes in dietary habits.[28] In addition, the infant's diet adjusts to meet the demands of growth and increasing energy expenditure.[29] Breast milk is gradually replaced by formula, while consumption of drinking water and food increase, becoming additional pathways for exposure to potentially toxic chemicals.

Children display upright posture consistently during preschool age. This can diminish exposure to chemicals that settle on low-elevation surfaces, although preschool-age children continue to have low stature and spend considerable time playing on the floor or ground. Upright posture can also extend the boundaries of their immediate environment, particularly as object handling, locomotion, and exploratory behaviors continue to develop.[25] At this stage, children consume a higher quantity of solid foods and show preference for particular food items such as fruit and dairy. Beginning with preschool age and continuing into child and adolescent years, new environments outside the home become potential sources of exposure as children often spend much of their time in day care, playgrounds, schools, and after-school settings. These can be thought of as child-specific exposure settings that can contribute to differential sensitivity to a number of chemicals.[30,31] The development of coordination, complex movements, and a wider variety of exploratory behaviors in older children and adolescents facilitate activities in outdoor environments, including hazardous sites. The workplace may be an additional source of exposure (e.g., solvents in industry, pesticides in farming) in adolescence, a period when children also undergo physiological changes associated with growth spurts, hormonal fluctuations, and dietary habits. Adolescents are more likely to adopt risk-taking behaviors and to make lifestyle choices that could lead to differential risks.

77.2.2.1.2 Breast-feeding

Breast-feeding is significant as a pathway of exposure to toxicants for children in early stages of development. The practice is strongly recommended during the first 6 months after birth and encouraged until the second year. Breast milk is a highly beneficial source of nutrients and other substances necessary for development, immunity, and disease prevention. These benefits generally outweigh potential risks from exposure to chemicals.[32] The risk–benefit balance may differ for specific populations, however, depending on known and likely risks associated with breast milk and its substitutes. For example, breast-feeding may not be a safe option for women living in areas with unusually high concentrations of lipophilic and persistent chemicals. Substances with these properties, such as dioxins and organochlorines, are favored for accumulation in breast milk.[33–35]

Chemicals pass from the maternal circulation to the mammary glands primarily by diffusion. The amount transferred depends of the compound's free fraction in maternal plasma, and also on its properties: substances that are low plasma-bound, lipid-soluble, of low-molecular weight, and weak bases are generally favored.[36–39] The properties of a chemical also influence transfer from breast milk to the newborn's or infant's circulation. The quantity transferred and ingested is partly determined by the composition of breast milk, i.e., the

fraction of lipids and proteins, but this varies by the day and timing of feeding, as well as within a feeding.

A small fraction of the maternal chemical dose is absorbed with a single feeding of breast milk, but cumulative transfer over the duration of breast-feeding is substantial. The contribution of breast milk consumption to a child's body burden differs by chemical. Studies have shown that the first 60–90 days of breast-feeding could contribute 36–80% of total blood lead, the first 6 months could transfer over 20% of some persistent pollutants such as polychlorinated biphenyls (PCBs), and one year could lead to doses of dioxin six times higher than nonbreast-fed infants'.[33,38,40] For neonates feeding on formula, exposure may involve a different set of substances and risks depending on the type and location of the water sources. Studies have shown that using water high in nitrates, nitrites, or aniline dyes can induce methemoglobinemia (or blue baby syndrome), since neonates and infants are susceptible due to enhanced absorption and differences in biotransformation of these chemicals.[41–43]

77.2.2.2 Biological Factors

77.2.2.2.1 Absorption: Gastrointestinal

Gastrointestinal function is immature and develops rapidly in the first month of life. The gastric environment is less acidic compared to an adult's, and this favors absorption of basic substances.[44] Relative to adult values, the small intestine is shorter with greater absorptive surface area, gastric emptying is prolonged and leads to delayed absorption, and transit times are shorter and may decrease absorption.[29,45–49] Pancreatic function and gastrointestinal motility are also immature, respectively limiting absorption of lipids, and leading to accelerated absorption or variable reductions in the dose absorbed.[29,47,50,51] Heavy metals are absorbed and retained to a greater extent at this stage.[52]

Several gastrointestinal processes mature in infancy but some continue to develop until the end of adolescence. Gastric pH, emptying rates, and pancreatic function reach adult levels by the first year of age.[42,44,51] In preschool years, the small intestine and transit times are shorter, gastric motility is faster, and absorption of lead is still greater than adults'.[21,29,45,53] Gastric emptying increases above adult levels at this stage, possibly resulting in faster absorption and higher peak serum concentrations but not necessarily a greater extent of absorption.[20,29,53] Emptying remains higher through childhood and motility remains faster through adolescence, though it is not clear how this affects the absorption of chemicals.

77.2.2.2.2 Absorption: Dermal

The skin's barrier function is thought to be nearly mature at the neonatal stage, and its thickness is less than that of older children's or adults'.[20,48,54] The stratum corneum—the outer layer of the epidermis—is fully developed at birth for neonates born at term. Passive diffusion across an intact stratum corneum is the rate-limiting step for dermal absorption, but topical agents must also traverse the lower epidermal layers before reaching the systemic circulation through the blood and lymph capillaries in the dermis. The structure and function of the epidermis reaches maturity by the second or third week of life. In addition to skin permeability, the surface area over which absorption takes place is also an important factor for dermal uptake of chemicals. Neonates' surface area to body-mass ratio is three times higher than adults' and can lead to greater systemic doses given the same amount of topical exposure.[19,29,52]

Although the structure and function of the skin are fully developed before the first post-natal month the surface area to body-mass ratio remains larger until the end of adolescence. As a result, systemic doses from dermal exposure may be greater for several years after birth.[21,55] This suggests persisting child–adult differences in the capacity to absorb chemicals through dermal contact.

77.2.2.2.3 Absorption: Pulmonary

Pulmonary system function is immature at the neonatal stage, given lower levels of several parameters including lung volume, parenchymal volume, number of alveoli, and alveolar surface area.[22,56,57] Neonates have high respiratory minute ventilation, which results in greater (weight-adjusted) inspired air per unit time.[55] This can lead to higher doses of some air pollutants such as particulate matter, but pulmonary absorption of chemicals is influenced by additional factors such as their solubility and blood flow.[58,59] Information is limited for these and other parameters potentially affecting differential susceptibility, such as pulmonary absorption, inhalation, and retention of chemicals.[23]

The structure and function of the pulmonary system continues to develop throughout infancy and childhood. Evidence suggests that alveolarization, alveolar surface area, and the number of alveoli reach maturity before adolescence, though the exact timing is difficult to determine.[22,55–57] Adult airflow patterns also emerge during that time, following the development of nasal passages.[60]

77.2.2.2.4 Distribution

The distribution of chemicals absorbed into the neonatal circulation differs from adult distribution due to chemical–protein binding and body composition. Newborns have qualitatively different and lower concentrations of plasma proteins, and higher concentrations of endogenous substrates such as free fatty acids and bilirubin.[6,20,48,61–63] The likely result of these differences is decreased binding and displacement from binding sites. This leads to higher circulating free fractions of a chemical. Newborns also have a high percentage of body water (75% of total weight). Combined, these factors suggest relatively greater distribution of chemicals (particularly hydrophilic and polar compounds), increased equilibrium concentrations at target organs, and enhanced potential for toxicity. The low percentage of body fat at this stage (15% of total weight) has another important implication for toxicity, as the brain continues to be a comparatively larger storage compartment and target organ for lipophilic chemicals.[55]

Serum albumin concentrations and affinity for substrates reach maturity by the first year of age.[20,48,63] The distribution of chemicals remains different in infancy, however, since total serum protein concentrations are comparatively lower and total body water is greater compared to adults. The level and function of binding proteins mature by the end of a child's preschool years.[20] Past this stage, protein binding does not contribute to child–adult differences in the distribution of chemicals. The percent total body water gradually decreases and body fat increases until adult values are reached around the beginning of adolescence. This suggests that the brain remains a major target organ for several years after birth.

77.2.2.2.5 Metabolism

Most metabolic functions are immature at the neonatal stage but develop rapidly at variable rates.[9,20,29] CYP450 and alcohol dehydrogenase, oxidative enzymes involved in phase I metabolism, have low concentrations and activity levels, while reductive enzyme systems are near adult levels at birth.[8,9,12,53,63] In addition, the neonate has limited capacity for glucuronidation, a phase II reaction which detoxifies a range of chemicals (particularly phenols, alcohol hydroxyls, carboxyl groups) and accounts for most substances excreted in bile or urine.[9,10] Acetylation, the major metabolic pathway for compounds containing aromatic amino or hydrazine groups, also functions at lower than adult levels.[10,64] Other phase II processes are not deficient at this stage. Glutathione conjugation reactions are thought to be at or near mature levels, and sulfate conjugation is a significant detoxifying pathway.[9,10,48,65] Overall, the metabolic rate of chemical substances dependent on these pathways is reduced, and their elimination is prolonged. The variable activity of metabolic enzymes may result in production of metabolites that differ from adults' qualitatively and quantitatively.

Phase I and phase II metabolic reactions mature before or during the child stage. The activity of CYP450 system enzymes reaches adult levels after the first year of life, and alcohol dehydrogenase activity continues to develop until the end of preschool years.[9,20,48,63] Most phase II reactions also mature between infant and child stages. Acetylation and glucuronidation reactions reach adult levels towards the end of infancy and in early preschool years, respectively.[64,66] The transition from sulfation to glucuronidation as a dominant metabolic pathway is thought to occur during preschool and child stages, resulting in the capacity to biotransform a greater range of chemicals.[10,20,48] In adolescence, metabolic function may be affected by hormone-induced changes in the liver.[30]

77.2.2.2.6 Elimination

Renal and biliary excretion functions are not fully developed in newborns. Bile acid metabolic processes are immature, leading to decreased intestinal absorption and biliary excretion of lipophilic or other compounds.[67,68] Glomerular filtration rates, which are proportional to renal clearance of chemical substances, are lower compared to adults.[69] Also functioning at reduced levels is renal blood flow, which determines the amount of chemical reaching the kidneys, and tubular function, which transports chemicals for renal excretion.[20,70] The result is inefficient elimination of chemicals and lower capacity to filter highly protein-bound circulating substances.

Excretory functions develop within a period of few months to reach maturity by the first year of age.[20,68,70] Beginning with infancy, the kidney concentrates urine similarly to an adult, and metabolized chemicals are efficiently filtered and eliminated.

77.3 DISCUSSION

77.3.1 Postnatal Patterns of Maturation

Biological processes that affect the absorption, distribution, metabolism, and excretion of chemicals mature at different times during development. Table 77.1 summarizes developmental trends and timing of maturation within and across these functions.

In the first month of life, most processes are immature and rapidly developing. During infancy there is a greater variation in maturational patterns, pointing to this period as one of the important developmental changes. Around 3 months but primarily between the fifth and twelfth months of life, processes of gastrointestinal absorption, distribution (binding proteins), and elimination reach adult levels. Important processes of phase I and phase II metabolism (CYP450, acetylation) gradually mature between the first and second years of age. Metabolic function continues to develop into the preschool and child stages when components of distribution and pulmonary function also reach adult levels. For other processes relating to absorption and distribution (body composition), maturation is reached gradually by the end of adolescence.

77.3.2 Implications for Susceptibility

Fetuses can be differentially susceptible to the effects of a toxic chemical due to characteristics unique to the prenatal stage. Their active and well-timed development makes exposure timing a critical factor, while their dependence on the mother brings them into contact with environmental chemicals (through concurrent exposure and mobilization of previously stored chemicals) and affects toxicokinetics (placental transfer, biotransformation, amniotic fluid recirculation). Lipophilic chemicals are of particular concern as their potential effect on the fetus is facilitated by a number of these factors. Lipophilic compounds are preferentially transferred across the placenta (as early as the fourth week of gestation), can be efficiently distributed to target tissues, are more likely to accumulate in the brain (due to low body fat and immature blood–brain barrier), and are not readily metabolized or eliminated. Some ubiquitous neurotoxic substances such as PCBs can be mobilized from fatty tissue during pregnancy, adding to the fetus' concurrent exposure levels.

Lipid-soluble chemicals are favored for transfer into breast milk, together with basic, poorly protein bound and

TABLE 77.1
Development and Maturation of Factors Affecting Toxicokinetics

	Developmental Trend	Timing of Maturation
Gastrointestinal absorption		
Basal acid secretion rates and gastric pH	Lower in neonatal, infant stages Higher during child stage	Infant: third month
Anaerobic bacteria colonization and flora acidity	Variable in neonatal, infant stages	Infant: 5–12 months
Gastric emptying rates	Lower in neonatal, infant stages Higher during preschool and child stages	Infant: 6–8 months
Pancreatic lipase activity, bile acid pool	Lower and variable in neonatal, infant stages	Infant: 5–9 months
Inorganic element absorption	Higher, until preschool stage at least	—
Small intestine	Shorter, until preschool stage at least	—
Gastrointestinal motility patterns	Faster and variable until end of adolescence	—
Gastrointestinal transit time	Shorter until end of adolescence	—
Dermal absorption		
Skin structure and function	Immature in neonatal stage	Neonate: 2–3 weeks
Surface area to body mass ratio	Greater until end of adolescence	—
Pulmonary absorption		
Number of alveoli and alveolar surface areas	Lower until preschool stage	Preschool and child: 2–8 years
Total lung and parenchymal volume	Small until mid-infant stage at least	—
Fraction of lung occupied by parenchyma	Smaller until child stage at least	—
Respiratory minute ventilation	Higher until end of adolescent stage	—
Nasal passage growth	Ongoing until child stage	Child: 6–12 years
Distribution—binding proteins		
Concentration of plasma protein	Reduced until preschool stage	Preschool: 2–5 years
Plasma protein content	Different until neonatal stage at least	—
Concentration of endogenous substances	Higher until early infant stage at least	—
Affinity of albumin for acidic chemicals	Lower until mid-infant stage	Infant: 10–12 months
Affinity of albumin for bilirubin	Lower until mid-infant stage	Infant: 5–12 months
Concentration of plasma albumin	Lower until mid-infant stage	Infant: 10–12 months
Distribution—body		
Total body water	Higher until adolescent stage	Adolescent: 12–13 years
Brain weight relative to body weight	Greater until neonatal stage at least	—
Metabolism: phase I		
Cytochrome P450 activity and function	Lower and variable until mid-infant stage	Infant: 10–20 months
Reductive enzyme activity	Mature in early neonatal stage	Neonate: at birth
Alcohol dehydrogenase activity	Immature until end of preschool stage	Preschool: 5 years
Metabolism: phase II		
Glucuronidation pathway	Lower until preschool stage	Preschool: 3 years
Acetylation capacity	Lower until preschool stage	Preschool: 2 years
Glutathione conjugation capacity	Mature in early neonatal stage	Neonate: at birth or soon after birth
Sulfate conjugation capacity	Higher until preschool stage	Preschool and child: 3–10 years
Elimination		
Glomerular filtration rate	Lower until early infant stage	Infant: 2.5–5 months
Renal blood flow	Lower until early infant stage	Infant: 7–8 months
Tubular secretion	Lower until early infant stage	Infant: 7–8 months
Biliary excretion	Lower until mid-infancy	Infant: 12–13 months

low molecular weight compounds. Since these properties also tend to favor transfer across the placenta, the same types of chemicals may be absorbed in both prenatal and early postnatal stages. The high lipid and protein contents of breast milk aid uptake into the neonatal circulation at a time when the processes of distribution, metabolism, and elimination are immature. This reinforces the mechanistic basis for susceptibility to lipophilic chemicals. Therefore, one could think of the prenatal and early postnatal stages as a single window of susceptibility to chemicals with lipophilic characteristics, particularly with respect to nervous system-related effects.

The absorption of heavy metals and other inorganic compounds is also pronounced at neonatal and infant stages. Since processes related to gastrointestinal absorption, distribution, metabolism, and elimination do not reach adult values before the first year of age, neonates and infants can

be differentially susceptible to such chemicals. This becomes increasingly important as breast-feeding gradually ceases and infants begin to consume drinking water, formula, and solid foods. In addition, certain chemicals may be ingested via nondietary oral intake associated with mouthing behavior, a pathway unique to young children. Overall, ingestion appears to be a significant exposure route during infancy, a period associated with immature gastrointestinal function and with changes in the type and variety of chemicals taken in.

Other exposure pathways become increasingly significant as movement and exploratory behaviors develop and children begin to spend more time outside the home (outdoors, day care, school, workplace, etc.). Exploration of new environments, risky behaviors, and hazards associated with them may introduce new dietary and nondietary exposures. Unlike gastrointestinal absorption, many processes related to pulmonary function do not reach adult levels until the latter stages of childhood. The potential for higher systemic doses from dermal exposure also continues until the end of adolescence, when surface area to body-mass ratio becomes equivalent to an adult's. While the elimination of chemicals reaches maturity during infancy, distribution and metabolic functions can contribute to differential susceptibility until adolescence.

77.3.3 Dermatotoxicity

The skin serves as a barrier between an organism's internal and external environments due to its very low permeability. Yet it is also a route of exposure, since certain substances can be absorbed via the dermal route to potentially reach the systemic circulation. Exposure through skin contact may contribute to the body burden of lipophilic and other chemicals in the environment.

Chemicals carried in water and adsorbed onto soil can be in contact with the skin for long periods of time. Exposure to pesticides such as chlorpyrifos may be disproportionate for children since they spend more time playing in soil. Similarly, uptake of chemicals dissolved in water may be greater, considering child–adult differences in the total-surface exposure when bathing and swimming. Lipophilic substances can be easily absorbed by dissolving into the lipid–protein structure of the stratum corneum and possibly forming a "reservoir" in the skin.[71] Metals and other chemicals dissolved in water may also be carried across the dermal barrier.[72] Percutaneous absorption is greater for part of the first postnatal month, and the body surface area to mass ratio remains larger for several years after birth, potentially leading to higher systemic doses. This represents a relatively long window of susceptibility for dermatotoxicity, whose significance in terms of risk would depend on additional factors such as the specific exposure and maturation of other pharmacokinetic functions.

77.4 CONCLUDING REMARKS

Children's sensitivity to a potentially toxic chemical is more likely to become apparent when elevated exposures or doses coincide with susceptibility, or "critical windows" of development. Both susceptibility and differential exposures are influenced by life stage-specific characteristics. A more complete understanding of these developmental patterns is needed to better assess the effect of toxic exposures on children's health.

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78 Utilization of Irritation Data in Local Lymph Node Assay

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78.1 INTRODUCTION

Lymph node cell proliferation has so far formed the basic paradigm of the local lymph node assay (LLNA) for the identification of contact allergenic potential of small molecular weight chemicals. Since the activation of specific effector lymphocytes and the formation of immunological memory are the hallmarks of a specific immune response depending on clonal expansion of specific lymphocytes, cell proliferation in the lymph nodes (LN) was consequently seen as an indicator of the sensitizing potential of a chemical. However, there is accumulating evidence on irritating chemicals, which cause false-positive results in the LLNA by inducing proliferative events in the skin-draining lymph nodes [1–6]. The complex mechanisms leading to irritant-related cell proliferation in the LN are not completely understood, but the phenomenon itself may lead to a reconsideration of the basic principles of generation and evolution of specific immune responses induced by chemicals with the potential to form hapten-carrier-conjugates.

Irritation is often realized as a confounding factor in animal or human testing for contact allergenic potential of low molecular weight chemicals. Thus, in the biphasic guinea pig tests it is important to treat animals with the lowest still irritating concentration to ensure a successful sensitization while avoiding unnecessary toxicity. Elicitation of contact allergy should be tested with the highest nonirritating concentration to identify a potential lowering of the reaction threshold typical for allergic reactions. In cases of chemicals with a high intrinsic irritant potential, these testing guidelines may become difficult to follow. Similar hurdles have to be taken in human patch testing, where irritating concentrations of sensitizers may result in false positive responses. Beside these practical difficulties arising from irritant potential of chemicals, there is a statistical relationship between irritancy and skin sensitizing activity [7]. Supporting evidence that the inflammatory response caused by irritation constitutes an important part of the sensitization process, leading to chemical contact allergy, was brought up in attempts to include skin inflammation endpoints into the LLNA [2,4]. Either the chemical, its solvent, or an additional chemical in a topical formulation can irritate the skin in a way that proinflammatory cytokines and chemokines are released by epidermal cells. This release forms the initiation of a cross-talk with compartmental immune cells like the Langerhans cells in the epidermis, which then migrate to the regionally draining LN where they present antigen to T cells [8]. Whether a skin sensitization can happen depends finally on the ability of the chemical to bind to self-structures and form immunogenic hapten-carrier-conjugates. The influence of irritation on contact sensitization had been first shown by Magnusson and Kligman [9], who increased the frequency of chemical-sensitized guinea pigs by applying sodium lauryl sulfate to the skin. Grabbe et al. [10] were able to show that sensitized mice could mount a challenge response to a suboptimal dose of the chemical allergen, when a chemically unrelated sensitizer was added at a concentration causing primary irritancy in the skin. Recently, Jacobs et al. [11] demonstrated in human skin explant cultures that skin irritation by both nonallergenic and allergenic chemicals induced LC migration and maturation underlining that general inflammation induced by irritancy is an important part of the sensitization process.

78.2 ASSESSING SKIN IRRITATION WITH THE LLNA

The first modification of the original LLNA, which utilized incorporation of radioactively labeled thymidine, was the establishment and validation of LN weight and cell counts

as endpoints [12]. These nonradioactive endpoints provided more flexibility for the LLNA by opening it for the use of additional endpoints like LN cell phenotyping by flow cytometry and determination of cytokine release [1,22,23]. The increasing evidence that pure irritants can cause positive LLNA results, facilitated the introduction of ear thickness or the weight of ear punches to directly relate skin irritation to the LN activation [2,4,13]. The establishment of a direct relationship between skin irritation and LN activation in one LLNA study ensures that no additional confounders like different species, different vehicles, or study designs, which are not comparable to the LLNA can hamper data interpretation. There are two methods to assess skin inflammation after chemical irritation in a reliable and easy-to-apply way: determination of ear thickness with a micrometer and the weight of circular skin samples from the apical area of the ear using analytical scales. Measuring ear thickness has the advantage of an in-life parameter, and can be used to establish a kinetic of ear swelling during the course of a LLNA study. Care has to be taken that in one study always the same person performs the measurement, because there is a subjective component influencing the handling of the micrometer, the position of the instrument sensors on the ear, and the time when reading the thickness value from the micrometer after placing the instrument sensor on the ear. The last point is crucial, since the fully developed instrument pressure on the ear will squeeze the tissue and thereby cause a continuous decrease in thickness. Ear weights, in contrast, can only be taken once after sacrifice of animals. The advantage, however, is the very low interindividual variation of ear weight data within the vehicle group, increasing the confidence in data from treated groups indicating skin irritation potential of the test chemical. Measuring ear thickness or weights can easily be implemented in a routine environment. Careful excision of the circular skin samples with a biopsy punch from the apical area of the ear is absolutely necessary. Disregard will lead to larger variation of individual weight data due to the different thickness of the mouse ear from the basis to the apical tip.

In an interlaboratory validation of the modified LLNA, ear thickness and ear weight were assessed together with LN weight as well as cell count [14,15]. With the exception of two laboratories ear weight data from vehicle-treated groups were within the same range, and all laboratories provided data with a remarkable low intralaboratory variation (Table 78.1). Ear weights were also demonstrated to reflect the differences in irritant potential exerted by the nonsensitizing irritant croton oil and the sensitizer oxazalone while both oxazolone and croton oil induced comparable LN hyperplasia at the applied concentrations [2]. In a kinetic LLNA study, it could be shown that croton oil caused a rapid increase in ear weights, whereas oxazalone produced a slower increase and was also less irritating (Figure 78.1). In a biphasic LLNA using the sensitizer oxazalone [2], the ear weight increase in the challenge group exceeded the changes in the induction control groups indicating that ear weight is a useful marker to demonstrate the increased reactivity in a sensitized animal (Figure 78.2).

TABLE 78.1
Results of Vehicle Controls (DAE433), Means and Standard Deviations

Laboratory No.	Cell Counts ($\times 10^6$ Cells/Animal)	Lymph Node Weight (mg)	Ear Weight (mg)
1	4.68 ± 1.40	4.93 ± 0.37	22.47 ± 0.31
2	9.71 ± 2.80	5.86 ± 0.60	20.45 ± 1.57
3	7.68 ± 1.47	4.10 ± 0.52	27.00 ± 1.41
4	6.33 ± 1.85	4.10 ± 0.73	19.05 ± 1.02
5	14.69 ± 2.89	6.05 ± 1.05	21.87 ± 1.16
6	9.59 ± 2.27	7.88 ± 1.70	22.40 ± 0.89
7	3.65 ± 0.94	4.13 ± 0.69	22.45 ± 1.08
8	4.60 ± 2.00	5.00 ± 1.00	23.80 ± 1.10
9	10.46 ± 2.13	6.30 ± 0.78	27.52 ± 4.48

Note: Data from an interlaboratory validation
Source: Ehling, G. et al., *Toxicology*, 212, 69, 2005. With permission.

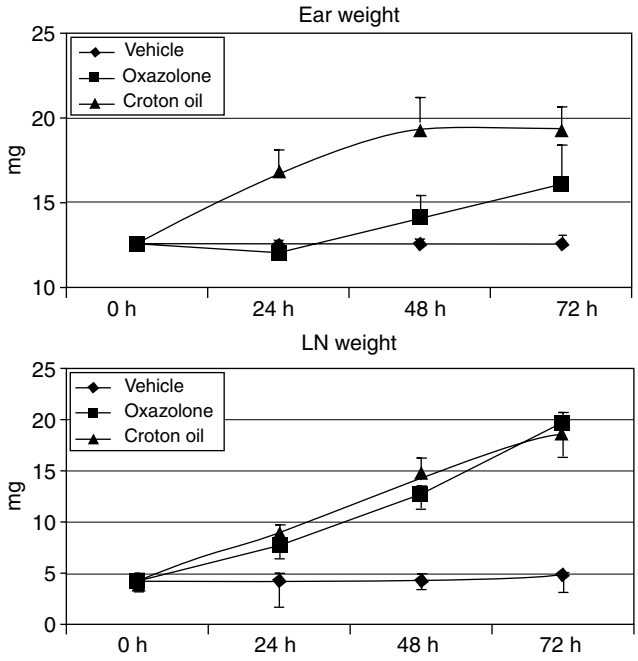


FIGURE 78.1 Kinetic of ear skin irritation and LN activation induced by the contact allergen oxazolone and the irritant croton oil. Six female BALB/c strain mice per group were treated on 3 consecutive days with chemicals on the dorsum of both ears. Twenty-four hours after each application of test chemical, the respective group of mice was sacrificed and ear weights and LN weights were determined. Values at "0 h" refer to untreated animals. Both croton oil and oxazolone were applied in DAE433 at 1% (w/v) and (v/v), respectively. Mean ear weights were computed using individual weights taken from circular pieces (0.28 cm^2) punched from the apical area of one ear. Mean LN weights were derived from pairs of auricular LN per individual animal. (Ulrich, P., Streich, J., and Suter, W. *Arch. Toxicol*., 74, 733, 2001. With permission.)

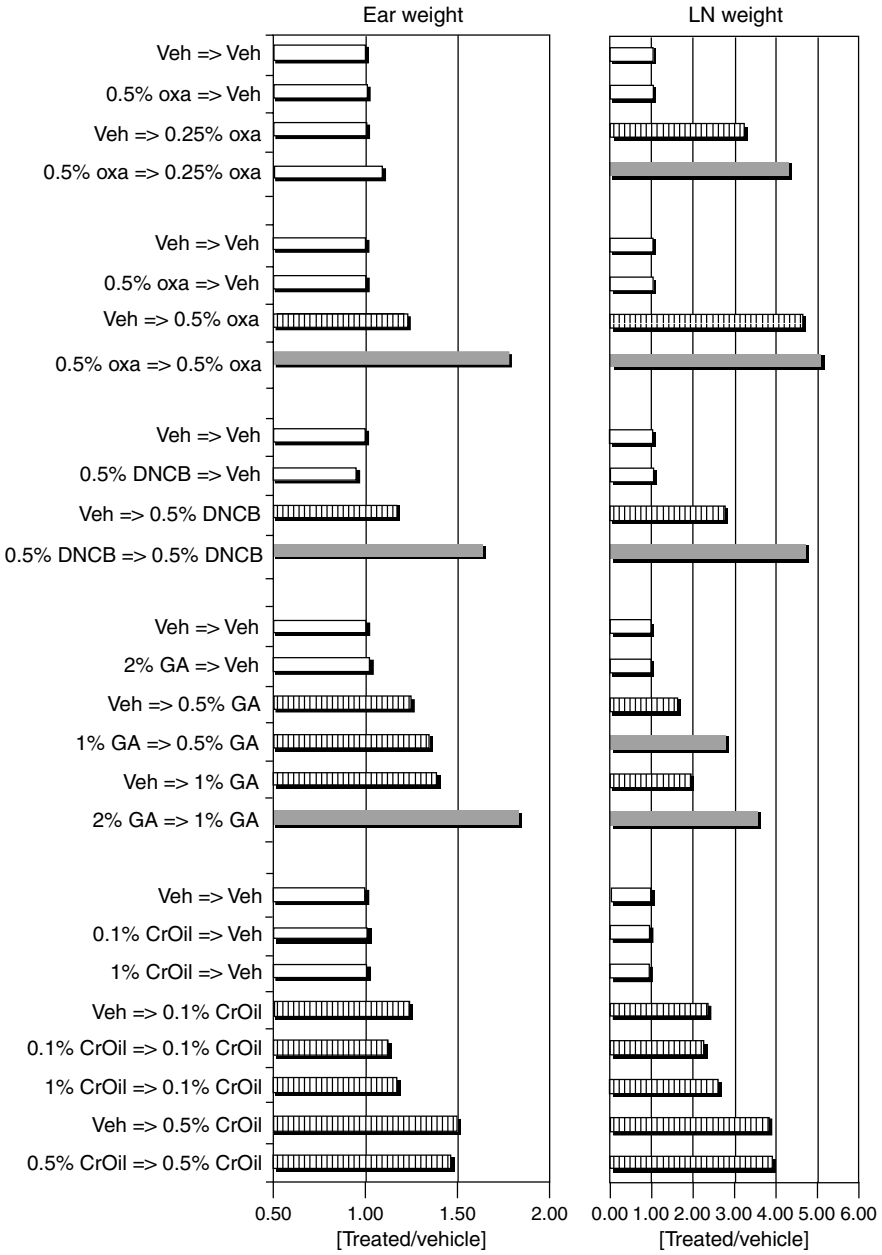


FIGURE 78.2 Challenge responses following secondary exposure to chemicals. Six female BALB/c strain mice per group were treated on 3 consecutive days with chemicals on the shaved back (dorso-lumbosacral). Twelve days after the induction phase treatment, mice were challenged on the dorsum of both ears for another 3 days (induction phase => challenge phase treatment). Twenty-four hours following the last exposure ear weights and LN weights were determined as described for Figure 78.1. Indices were built from treated groups versus the vehicle control with an index set to 1. Statistical analysis was performed by comparing chemical-treated groups and the corresponding vehicle controls (striped bars: $P < 1\%$), as well as between challenged groups and the corresponding induction control groups (filled bars, $P < 1\%$). (oxa: oxazolone; GA: glutaraldehyde; Cr Oil: croton oil; Veh: vehicle; DNCB: dinitrochlorobenzene) (Ulrich, P., Streich, J., and Suter, W. *Arch. Toxicol*., 74, 733, 2001. With permission.)

78.3 RELATIONSHIP BETWEEN IRRITATION AND CONTACT SENSITIZING POTENTIAL

A large set of known standard sensitizers and irritants was tested in a modified LLNA with ear weights and LN weight, as well as cell counts as endpoints [2]. A similar approach using ear thickness was reported by Vohr et al. [4]. To compare the potencies of the chemicals to induce LN hyperplasia and skin irritation threshold indices were derived from historical data sets. Figure 78.3 plots the concentrations to overcome the threshold index for LN hyperplasia against the respective concentration to overcome the skin irritation threshold index. The plot shows that weak contact sensitizers like mercaptobenzothiazole (MBT), cinnamic aldehyde, or isoeugenol appeared as weak inducers of LN hyperplasia and showed a weak or no skin irritation potential in the LLNA. The standard irritant sodium dodecylsulfate (SDS) was located slightly above these weak sensitizers with almost

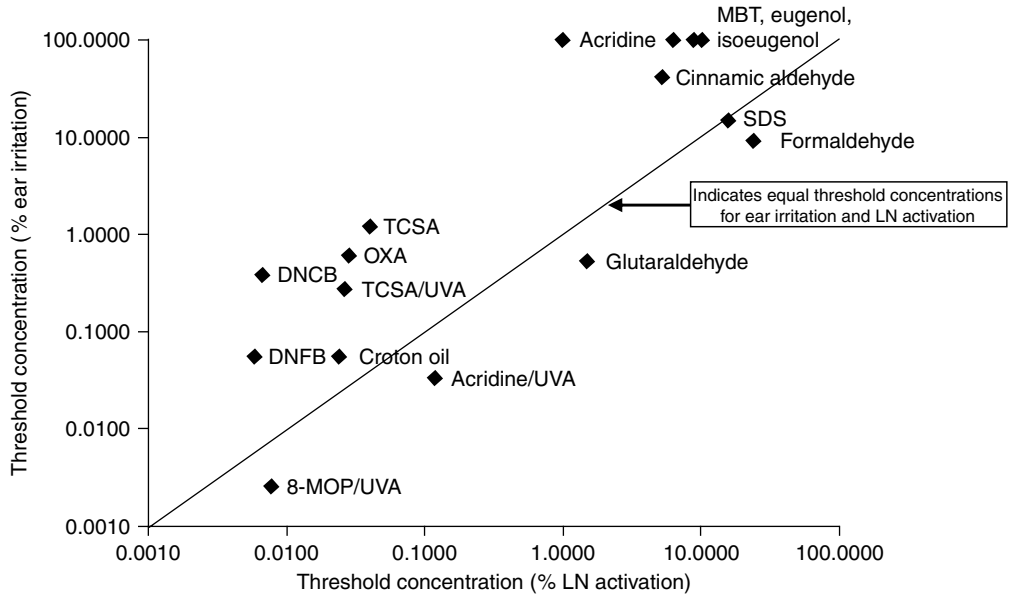


FIGURE 78.3 Relation of primary ear skin irritation and LN activation induced by chemicals. Threshold concentrations for primary LN activation and ear irritation were calculated for each chemical by applying curve-fitting algorithms to the concentration–response curves Tables 78.2 and 78.3. The threshold indices for LN activation (1.3) derived from cell count data and ear irritation (1.1) assessed by weight measurement were approximated from the lowest applied concentrations of the chemicals leading to statistically significant responses. To support the definition of threshold concentrations, a large set of historical data was included in the survey. (TCSA: tetrachlorosalicylanilide; UVA: Ultraviolett A) (Ulrich, P., Streich, J., and Suter, W. *Arch. Toxicol.*, 74, 733, 2001. With permission.)

identical threshold concentrations for LN hyperplasia and skin irritation. It is noteworthy that all contact sensitizers with a considerable potential to induce LN hyperplasia as oxazalone, dinitrochlorobenzene (DNCB) or dinitrofluorobenzene (DNFB) displayed a marked skin irritation potential. However, also nonsensitizing chemicals like the photoirritant methoxypsoralene 8-(MOP) and the irritant croton oil appeared in this group of chemicals with a marked potential to induce both LN hyperplasia and skin irritation.

Glutaraldehyde is known to have sensitizing potential, which can be attributed to its capability of covalent binding to various surface proteins. This behavior also represents the reason for its irritation potential, which, at high concentrations, may override clinical manifestation of allergy in the skin. The skin irritation potential of glutaraldehyde in the LLNA determined by ear weight occurs at lower concentrations in comparison to those necessary for the induction of LN hyperplasia [2]. Therefore, additional information would be necessary to correctly classify a new chemical in a routine situation with respect to skin sensitization. If there is no evidence from structural considerations that a chemical can cause contact sensitization, or if the primary LLNA gives equivocal result with respect to the specificity, a biphasic LLNA may be conducted. In such a biphasic LLNA, sensitization to glutaraldehyde was achieved with concentrations causing moderate to marked irritation [2]. Elicitation of contact allergy in the ear skin and secondary LN hyperplasia was achieved with a combination of 2 and 1% for sensitization and elicitation, respectively. However, significant challenge-related increases in LN weights, but not in ear weights, were also observed with 1 and 0.5% during sensitization and elicitation indicating that the threshold concentration for manifestation of glutaraldehyde-induced ear skin *allergy* is higher than the threshold for secondary LN activation (Figure 78.2).

Croton oil represents an example on how pharmacologically active chemicals may interfere with the endpoints of the LLNA. Croton oil contains phorbol esters and is a strong inducer of skin irritation and LN hyperplasia. In the lymph node, phorbol esters activate lymphocytes by specific interference in signal transduction via their protein kinase C-activating potential rather than by providing a specific antigenic stimulus [16]. Again, ear weight data provide additional information to design a biphasic LLNA to finally clarify the nature of croton oil activity in the LLNA. In such studies no contact allergic potential could be identified at different combinations of sensitization and elicitation concentrations, all of which caused primary changes in skin and LN (Figure 78.2).

An interesting case highlighting the crucial relationship between skin irritation and sensitization is the cationic surfactant benzalkonium chloride (BC). BC is a known irritant and in rare cases it can be a sensitizer. The diagnosis in human patch testing is often hampered by the marked irritant potential of BC and thereby increasing the risk of misinterpretation [17]. When tested in the modified LLNA, BC produced a bell-shaped concentration response curve for LN hyperplasia with a peak at 2% and a substantially lower value at 10% (Figure 78.4). Regarding skin irritation as assessed by ear weights, BC caused a positive concentration–response relationship up to highest tested concentration of 10%. Corroborating results were reported by Woolhiser [18] showing

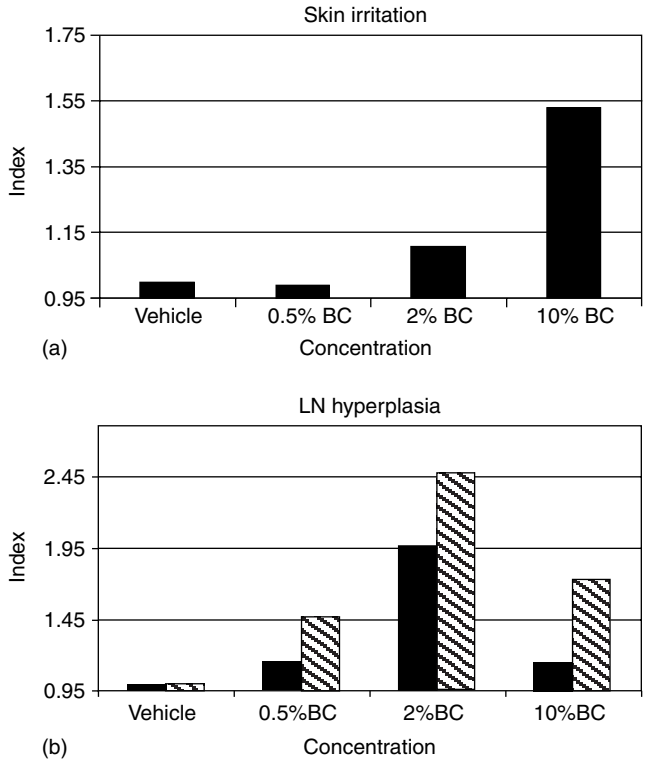


FIGURE 78.4 Concentration response for benzalkonium chloride (BC) in the LLNA. Six female BALB/c strain mice per group were treated on 3 consecutive days with chemicals on the dorsum of both ears. Twenty-four hours following the last exposure ear weights and LN weights were determined as described for Figure 78.1. Indices were built from treated groups versus the vehicle control with an index set to 1. Filled bars in the lower graph represent LN weight indices, whereas striped bars show LN cell count indices. (Unpublished data from Ulrich.)

a bell-shaped dose response for LN cell proliferation peaking at 2% in an LLNA and a positive concentration–response relationship up to 5% in a mouse ear swelling test (MEST). From these results, it is clear that BC bears a considerable irritant potential, which is—along the concentration range—inversely related to LN hyperplasia. The underlying mode of irritant action seems to be different from other sensitizing and nonsensitizing chemicals, which often show a direct correlation between skin inflammation and LN activation. However, there are reports providing evidence for a sensitizing activity of BC in biphasic guinea pig models [17]. Maurer [19] was able to elicit a contact allergic response in his guinea pig optimization test either after 0.1% intradermal challenge or after a 10% epicutaneous challenge with 55% positive responses in the first and 21% in the second test set up. The overall conclusion derived from the modified LLNA and the information from other animal tests as well as the human situation is that the foremost activity of BC is that of a skin irritant. In certain, obviously rare cases BC can act as a sensitizer and this may be indicated by the positive LN response at lower concentrations in the LLNA. However, the mechanism behind this inverse reaction pattern in skin and LN remains obscure.

78.4 PHOTOIRRITATION TESTING WITH THE LLNA

In former studies, the suitability of the modified LLNA to test for photosensitizing potential of chemicals had also been demonstrated [1,12]. In a further validation of this UV-LLNA, the introduction of ear skin irritation data in the UV-LLNA study protocol revealed that assessment of photoirritation provides additional help in determining the nature of the skin reaction—photoallergy or photoirritation [2,4]. In addition to epicutaneous administration of chemicals, oral application of test chemicals is also possible and once the chemical is distributed into the skin, the exposure of the mice to a sunlight simulating light source may induce a photosensitization. Chlorpromazine produced different patterns of skin photoreactions and related LN hyperplasia depending on the route of administration. After the oral route chlorpromazine produced significantly more skin photoirritation than LN hyperplasia, whereas after topical application, the LN hyperplasia was more in the foreground [4]. A comparable pattern of reactions depending on the route of administration is also observed in humans. The authors concluded that this route-dependent difference in the reaction pattern in the UV-LLNA may reflect a different subcellular distribution pattern of chlorpromazine. After oral application, chlorpromazine tends to distribute more into the nucleus of cells, which results in photon-induced DNA damages and hyperpigmentation of the skin, like with the psoralen 8-MOP. However, after topical application, the partners of photon-induced reactions are more likely proteins on the surface or the cytosol of cells, which then form hapten-carrier-conjugates with chlorpromazine leading to contact photoallergy. As a logical consequence one can observe a more pronounced LN hyperplasia after epicutaneous application of chlorpromazine.

To clarify whether a chemical is a photoallergen, the assessment of ear weights in biphasic UV-LLNAs becomes an important control in addition to the evaluation of skin reactions as described earlier for contact allergens. Local epicutaneous application of highly lipophilic chemicals leads to rapid systemic distribution, which can last for several weeks [1,2]. It was reported that more than 2 weeks after topical administration of 8-MOP on the shaved back of mice, elicitation of a photoirritation was possible simply by exposing animals to UVA light without further exposure to the chemical. It is obvious that such an effect, when not carefully controlled, will confound the comparison of skin reactions in animals challenged with chemical and light exposure with those receiving the treatment the first time.

78.5 CONCLUSION

Assessment of skin irritation in contact allergy and photoallergy testing with the LLNA has become an important endpoint, which helps to clarify the nature of the reactions observed in this assay. Since induction phase tests like the LLNA can detect fundamentally both pure inflammation

by chemical irritancy and allergy-relevant changes in skin-draining LN, it is important to detect the degree of irritation and establish the relation to the proliferative events in the LN. However, even with the addition of skin irritation assessment as an endpoint in the LLNA, it will still be necessary to use information from structure-activity-relationship databases to confirm the hypothesis of a putative contact allergenic potential. There is a fundamental consideration that only a positive result from these in-silico approaches can be used, since a negative result constitutes a null hypothesis. In cases of uncertainty or lack of a structural alert, it is recommended to conduct a biphasic LLNA to finally clarify the mode of action in mice—contact allergy or irritation. Both reactions, when investigated in the early phase, share many features on the histological level [20]. It is also evident that irritants activate a cascade of events with large similarities to those observed during sensitization to contact allergens without leading to a specific activation of T cells [20]. Recently, Vohr and Ahr [21] showed that they were able to reduce the number of positive LLNA results by introducing skin irritation assessment to a level comparable to positive rates obtained with guinea pig assays. Collectively, skin irritation assessment by using reproducible endpoints should be routinely incorporated in every LLNA. They can help with the interpretation of the results of the LLNA, but they cannot overcome completely some natural limitations of induction phase tests.

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79 Air Bag Injuries

Monica Corazza, Maria Rosaria Zampino, and Annarosa Virgili

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Air bags are automatic supplemental restraining safety devices, installed in motor vehicles; these devices are designed to fully inflate after a crash creating a protective cushion between the driver and the steering wheel and windscreen. Air bags therefore provide further protection for belted front-seat occupants in moderate-to-severe frontal or near-frontal crashes.

Air bags are not planned to inflate in side, rear, or rollover crashes; however, if forward deceleration of the car is sufficient, sensors may activate air bag deployment. More recently, side-impact air bags have been introduced to provide supplemental safety benefits during side-impact crashes. Side air bags are smaller and use less propellant than front air bags, but deploy very rapidly.

79.1 HISTORICAL AND EPIDEMIOLOGICAL DATA

Inflatable devices were patented for the first time during World War II for airplane pilots to reduce the impact during a crash; they were inserted in their life jackets.

The first civil air bag was patented in 1953. The mid-1970s saw air bags being installed as an optional extra by many car manufacturers in the United States, an innovation that reached Europe by the 1980s. By the 1990s, these devices were very common. Dual air bags were made a legal requirement on all new passenger cars in the United States after 1997. This was extended to vans, trucks, and utility cars in September 1998.[1–3] By 1999, it was estimated that 45% of cars and 41% of trucks in the United States were equipped with air bags.[4] Nowadays most cars contain air bags. By 2003, air bags in vehicles, solely in the United States, were estimated to total 257 million. Deployment was estimated to be 6.6/1000 air bags/year: that meant 1.7 million deployments in 2003.[5]

Different reports document the life-saving capability of air bags. A 50% reduction in mortality rates for motor vehicle accidents, when the driver is properly seat-belted, is estimated.[6] According to one statistical comparison, having an air bag in addition to a seat belt reduces the driver's death in frontal motor vehicle crashes by 28%.[7] The National Highway Traffic Safety Administration (NHTSA) reports that with the use of air bags, the risk of fatal injuries was reduced by 31% (http://www.nhtsa.dot.gov/airbag). In 2003, it was estimated that 2488 lives were saved by air bags.[8]

There is no doubt that air bags save lives. However, air bags that malfunction (oversized air bags, over-rapid deployment, or low-deployment threshold) or having defective electrical systems may cause injuries. Furthermore, a great deal of more or less severe injuries may be directly related to correct deployment or deflation.

An in-depth retrospective review for the years 1980–1994 reports 618 injuries resulting from air bag deployment. However, most of the injuries (96.1%) were considered of minor severity, whereas 2.9% were moderate, 0.8% serious, and only 0.1% critical.[1]

Even if severe and fatal injuries represent a small number in comparison with the saved lives in motor vehicle crashes, 169 deaths attributable to deployment of air bag have been registered by the NHTSA till 2000. In some cases, the speeds at which these crashes happened were so low that without an air bag only minor injuries would have occurred.

Paradoxically, air bag–induced injuries are more frequent and serious in low-severity crashes, especially in women.[3] Advances in sensor technology, depowering of air bags, reducing the explosive charge, installing on-off switches, and the introduction of smart air bags (which deploy at different speeds and forces depending on the occupant's physical size) will probably improve this crucial point. Since 1998, car manufacturers have been legally allowed to reduce the power of deployment to reduce serious injuries.

Injuries due to air bag deployment may cause damage to the eyes, ears, abdomen, chest, bones, and nervous system, as well as to the skin. Therefore, it is possible that several specialists may be involved in treating the patient in the emergency unit.

79.2 MECHANISM OF ACTION

Air bags are rubber-lined nylon bags folded into the center of the steering column but sometimes located in the side of the front seats; they may also be situated in the outboard edge of the seat back, in the door, in the roof rail above the doors, or in the pillars.

There are three steps in air bag activation: detection, inflation, and deflation.

In the first phase, crash sensors, located either in the front of the vehicle or in the passenger compartment, or both, detect a sudden longitudinal deceleration due to a rapid frontal or near-frontal impact. Triggering of the sensors activates a pyrotechnic device containing about 70 g of sodium azide; this propellant ignites and, via a series of exothermic reactions, releases nitrogen gases, which immediately inflate the bag (second step). Inflation occurs in about 10–50 ms at a very high average speed (about 150 mph), under high pressure, reaching a volume of about 30 (most European models) and 70 (some U.S. models) L. In addition to nitrogen gas, many other by-products such as carbon dioxide, metallic oxides, sodium hydroxide, to name but a few are also released creating a highly corrosive alkaline aerosol.

As sodium azide is a highly reactive substance, it may react chemically with water. This leads to the production of toxic and explosive products. In the presence of sparks coming from electrical devices or high temperature, it may ignite, causing thermal burns. Talcum powder and cornstarch, used in packaging the device, may also be released in the car.

The third step is the rapid deflation of the air bag (1–2 s). Gases are vented through exhaust ports or porous panels, normally situated at the eleven and one o'clock positions behind the restraining device.

79.3 SKIN INJURIES

79.3.1 Traumatic Lesions

Drivers of short stature, seated close to the wheel, and unbelted occupants are most at risk of traumatic lesions; lesions are caused by the rapid inflation of the air bag, which hits the face or the chest (barotrauma). The inflated air bag can also trap the hands and forearms between the steering wheel and the air bag itself. Secondary traumatic injuries may also be caused by other objects such as spectacles that break and cause nasal fractures and lacerations.

There is a high percentage of cutaneous traumatic lesions: abrasions, contusions, and lacerations represent, respectively, 63.6, 37.8, and 18.2% of the specific injuries observed in a retrospective study.[1] Traumatic lesions may be superficial self-healing abrasions or deep and large lacerations requiring sutures.

The air bag unfolding at high speed has a slapping action, which may cause numerous superficial parallel lesions on the face, chin, and neck. These quite typical linear erythematous and erosive lesions are defined as "friction burns."[9–13]

79.3.2 Irritant Dermatitis

The causes of irritant dermatitis are the mixture of gases, by-products of the combustive material, abrasive powders, and even talc released under pressure. Concomitant traumatic erosions may increase exposure of the skin to irritants worsening their damaging effects.[14]

Irritant dermatitis usually affects the face, arms, and upper chest. It is characterized by erythema with purpuric aspects, swelling, and sometimes blistering.[14,15] Patients complain of burning and stinging sensations on the affected areas. Irritant contact dermatitis is often superficial and usually resolves in a few days with desquamation, pigment discoloration, or postinflammatory hyperpigmentation as common sequelae.[14,15]

No cases of allergic contact dermatitis caused by allergens released during air bag activation have been found reviewing the current literature.

79.3.3 Thermal Burns

Thermal burns represent only 7.8% of all air bag–related injuries.[1] These lesions were sustained by 1.53% of front-seat occupants when air bags were deployed, according to a recent study in the United States covering the years 1993–2000.[16]

Thermal burns may be directly caused by high-temperature gases or may be indirectly induced by other objects.[9,17]

Burns occur most frequently when the extremely hot air that is vented through the ports during deflation is forced directly onto the skin.[18] Chemical products exploding on contact with electrical wiring also directly cause thermal injuries.[9]

A new category of direct burns, called "contact burns" from high-temperature bag, restricted to the center of the face, caused by a brief contact with an overheated bag, has been recently reported.[19,20] Mathematical models have been studied to predict the likelihood and severity of these types of burns; in particular, direct-contact burns are more likely to occur in case of delayed air bag deflation or prolonged contact exposure with the air bag.[5]



FIGURE 79.1 Burnt clothing resulting from high-temperature gases released during air bag deflation (Courtesy of Dr Massimo Nacca).



FIGURE 79.2 Superficial thermal burns to the forearm.

Full-thickness burns to the chest and hands are often indirectly caused by melted synthetic fabrics, especially lightweight ones, and overheated metallic accessories (Figure 79.1).[17]

Depending on the location of the exhaust vents (front or rear of the bag), face, arms, hands, and chest are the most frequently affected areas; in pediatric patients, lower extremities may be also involved.[21] Thermal burns may be superficial or of partial thickness.[9,22] Superficial burns are usually slightly erythematous (Figure 79.2); different-sized painful blisters and the detachment of superficial injured skin layers produce an exudative moist surface (Figures 79.3 and 79.4). Superficial burns usually resolve in a few days, sometimes leaving hypo- or hyperpigmentaton or atrophic wrinkled surfaces (Figure 79.5). Full-thickness burns are usually whitish, dry, with well-circumscribed wound margins; they are usually symptomless. In the case of direct-contact burns, the lesions have been described as well-circumscribed superficial dermal burns of the face with a fine blister membrane covering the raw surface[19,20]; the surrounding area is spared and no signs of contusion or lacerations are found.

Hot gases, ejected under pressure during deflation, may cause thermal burns to the hands; these have been described as "cigarette-like burns," sometimes with blisters.[11]

In the vast majority of burns, a combination of both chemical and thermal damages may occur, and some authors have suggested that a synergical effect may even lead to the development of full-thickness burns.[23]

79.3.4 Chemical Burns

Chemical burns are uncommon. The ignition of pyrotechnic device releases an alkaline aerosol consisting of sodium hydroxide, sodium carbonate, ammonia, and other alkaline by-products. These substances cause chemical burns.[11,24,25] When the aerosol solubilizes in liquids such as water, sweat, and tears, it has the most damaging efect.[10,15,25] The skin has a low capacity to buffer alkalis, which penetrate and induce deep-tissue injuries. Recent model air bags do not produce such strong alkalis; therefore, chemical burns are expected to be seen less frequently.

Chemical burns may sometimes be present as superficial painful areas of red-purplish erythema and edema of the face, chest, and arms.[24,26] Partial-thickness or even full-thickness chemical burns may occur when the skin is more deeply injured. They appear as well-demarcated areas sometimes with streaks or showing a splash shape.[23–25] In the case of



FIGURE 79.3 Partial-thickness burn to the forearm with large abrasions.

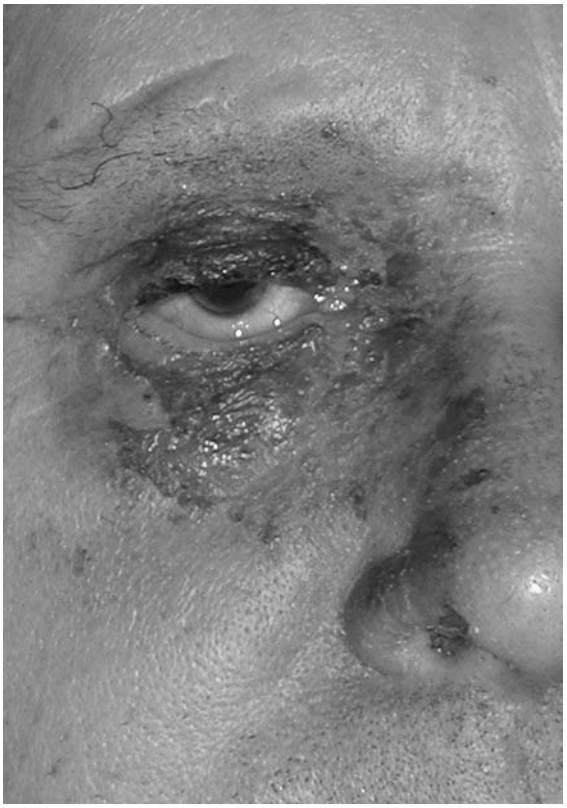


FIGURE 79.4 Facial thermal burns in a driver (Courtesy of Dr Massimo Nacca).

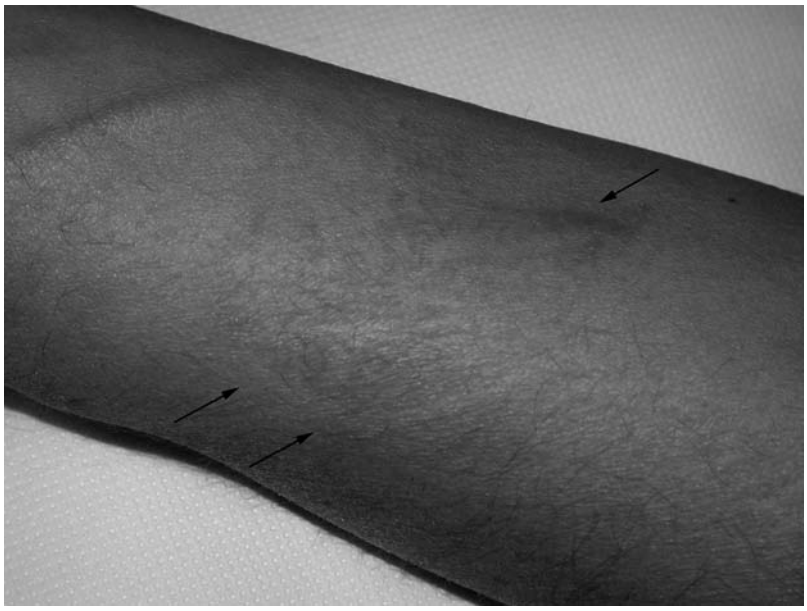


FIGURE 79.5 Atrophic wrinkled scar on a limb after thermal burns.

superficial lesions, differential diagnosis with irritant dermatitis may be impossible.

79.4 EYE INJURIES

Of all ocular injuries in motor vehicle crashes, 4.4% were associated with air bag deployment, according to a retrospective study.[27] In crashes with air bag deployment, only 5% of those involved had eye lesions. On the contrary, 12.7% of the eye injuries were found in crashes without air bag deployment.[27] Another study reported that 3% of all occupants exposed to an air bag deployment suffered an eye injury, whereas of those occupants who were not exposed to an air bag deployment, 2% had eye lesions.[28]

Air bags may represent a significant risk for eye injuries in slow-speed crashes, given that the higher rate of severe ocular lesions has been underlined.[6]

A recent study on the effects of depowered air bags on eye injuries revealed that of the occupants exposed to full-powered air bags, 3.7% experienced an eye injury, whereas only 1.7% of occupants exposed to depowered air bags sustained eye lesions.[29]

Air bag deployment–related eye injuries may be traumatic and chemical.[2] Thermal ocular damage is rare. Generally, eye injuries are monolateral, but bilateral lesions are not uncommon.[4,30]

The majority of traumatic eye injuries are due to the inflated air bag slapping against the eyes and the periocular tissues. However, air bags hitting drivers wearing spectacles at the time of the crash sometimes cause penetrating lesions, corneal lacerations, and various eyelid traumas.[30,31]

Eyelids, conjunctiva, and cornea are the most commonly involved ocular structures.[2,4,30,32] Rapid transfer of energy, followed by a rebound effect, can deform the ocular globe, leading to fixed ocular structures being stretched and damaged.[4,30] Numerous ocular injuries are reported in the literature: lens dislocations, retinal detachment, vitreous hemorrhages, traumatic iritis, intraretinal and subretinal hemorrhages, hyphema, angle recession, and even rupture of the globe.[32]

In children, the most frequently reported lesions are corneal abrasions.[33]

Air bag deployment generates the fine alkali aerosol that is the direct cause of ocular alkali chemical burns (chemical alkali keratitis).[13,34,35] In addition, the damaging effect may be prolonged by some alkaline substances depositing and crystallizing in the fornices. The referred symptoms are redness, lacrimation, burning, photophobia, and reduction of visual acuity. Permanent opacification and visual impairment may result from alkali burns. A case of Descemet's membrane detachment as a complication of a severe corneal alkali burns has been reported.[36] Spectacles may be useful in protecting the eyes from chemical injuries.

When an ocular alkali burn is suspected, ocular pH must be promptly monitored: ocular pH above 8, measured with pH paper in the lower fornix, confirms the diagnosis of alkaline keratitis.

79.5 EAR INJURIES

From telephonic interviews of drivers and passengers involved in "air bag" automobile crashes, it was presumed that hearing loss was an infrequent event (1.7%).[37] However, several case reports of hearing loss after air bag deployment are described in the literature.[38,39]

An air bag impacting on the side of the patient's head may induce various ear lesions such as external ear lesions, tinnitus, and disequilibrium.[37,40]

Barotraumas have been believed to cause a permanent threshold shift.[39,40] Air bag deployment generates a brief, high-amplitude pressure wave (impulse noise). With a driver-only air bag, the sound pressure is 159 dB. Dual air bag generates 165–170 dB of impulse noise. Therefore, the introduction of side and rear air bags increases the potential for ear injuries.[38]

There is no higher risk of developing hearing defects from air bag deployment for people with preexisting sensorineural hearing loss.[37]

79.6 MISCELLANEOUS INJURIES

Although severe or fatal air bag–related injuries are rare, they can include cerebral contusions, intracranial hemorrhages, brainstem transection or dislocation, fracture of the cervical spine, spinal cord injury, atlanto-occipital dislocation and, due to sudden forced hyperextension of the head and neck, base-of-the-skull fracture.[8,41]

Air bag–related death occurred in unrestrained or incorrectly positioned occupants, children, or small-statured persons, frequently women, who keep a full-forward position, which exposes them to a closer impact with air bag.

Bone fractures have been reported very often. Almost every part of the body is involved: ribs, clavicles, sternum, vertebra, radius, ulna, phalanx, and even thyroid cartilages.[8,42–44] Hand and wrist bone fractures, dislocation of the thumb, and even digit avulsions represent a further complication.[45] Elderly people are more at risk of bone fractures due to osteoporosis.[46]

Following deployment of the passenger air bag, facial trauma, temporomandibular joint injury, and one case of paresis of the facial nerve have been reported.[42,47,48]

Thoracic injuries of significance are rare; however, both steering wheel assembly and air bag can cause rib fractures and intrathoracic injuries; both hemothorax and pneumothorax have been described.[49]

Air bag deployment may also damage the cardiovascular apparatus: cardiac rupture (atrial and ventricular), contusion, and valve (tricuspidal and aortic) injuries have been reported.[8,42,50–52] Transections of both the ascending and the descending aorta, rupture of inferior thyroid artery,[53] and one case of a cutaneous fistula, secondary to foreign body reaction from a retained epicardial pacing wire in a heart transplant patient, have been observed.[54–56]

The release of irritating powders and gases may provoke respiratory problems such as coughing and asthmatic reactions in 10% of drivers and 15% of passengers involved in car accidents with air bag deployment.[57–59]

Children are more often subject to upper cervical spinal and head injuries especially due to incorrect use of seat belts and shoulder harnesses.[60,61] Children up to 14 years of age may be at risk for severe injuries when seated in front of the passenger air bag. Older children (aged 15–18 years), on the contrary, may benefit from the protective effect of the air bag.[62]

79.7 MANAGEMENT

The choice of the correct management of cutaneous lesions depends on the cause of injuries (Figure 79.6).

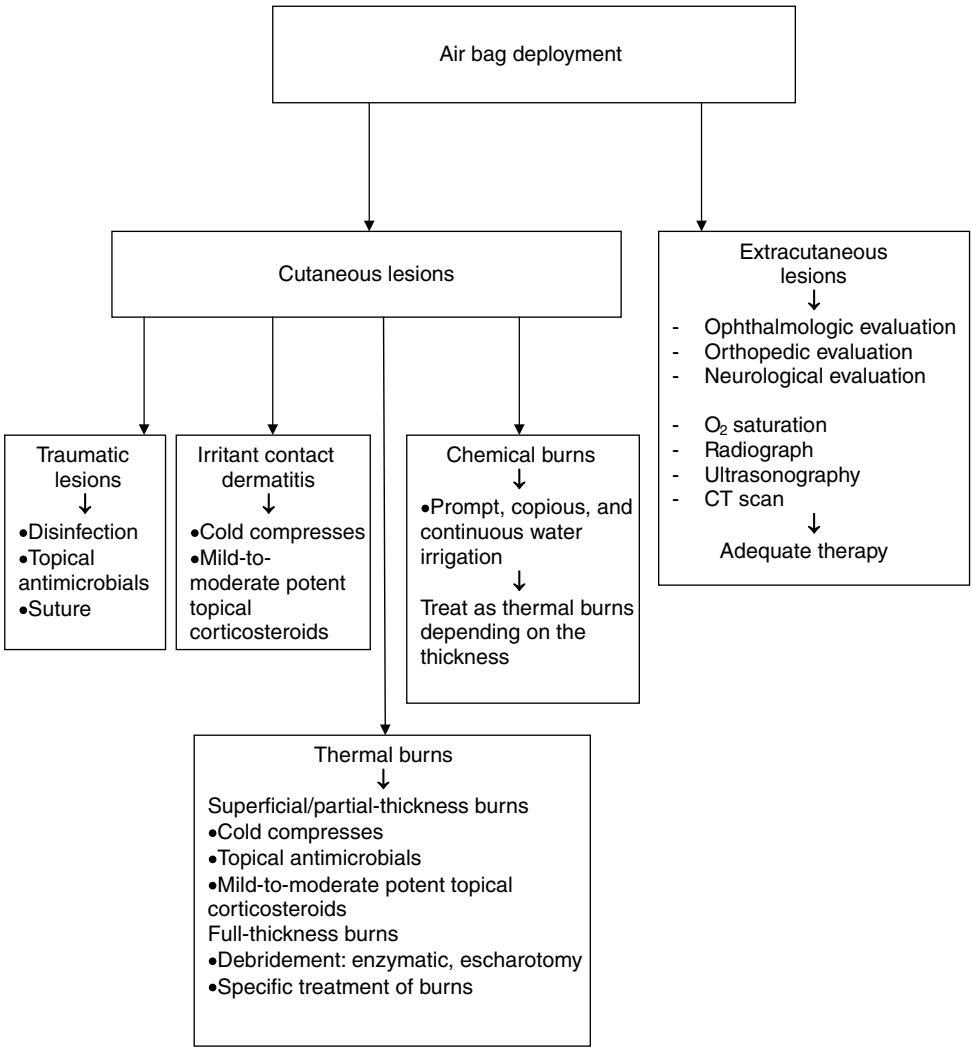


FIGURE 79.6 Algorithm: Management of air bag–related cutaneous lesions.

In the case of irritant dermatitis, application of mildly/moderately potent topical corticosteroid ointments (hydrocortisone 1%, clobetasone butyrate 0.05%) twice daily reduces inflammation and leads to rapid clinical improvement. Irritant dermatitis may only rarely require systemic corticosteroid therapy for a few days.

Patients affected by chemical injuries must be treated with prompt irrigation with cold or tepid water to avoid prolonged contact with chemical substances. It is essential to reduce the contact times with irritants as their concentration and delay in initiating the treatment can affect the depth of necrosis.

Correct evaluation of the depth of injury is essential for patients sustaining thermal burns to actuate the most appropriate treatment. Superficial thermal burns are easily treated with cold saline water compresses and topical corticosteroid therapy. Partial-thickness burns with blisters or abraded areas may benefit from topical antibiotics like gentamicin and bacitracin, applied under sterile dressings.

Full-thickness burns may require surgical debridement, with removal of necrotic tissues. After removal of necrotic eschars, the wound must be occluded to avoid superinfections and desiccation. Paraffin gauzes, synthetic inert membranes (silicone, polyurethane), or biological tissues (collagen) must be used to occlude the burnt areas. Silver sulfadiazine or topical antibiotics with a broad antibacterial spectrum are strongly indicated in local wound care.

Instructions must be given to patients to avoid direct sunlight and to use a topical broad-spectrum sunscreen to prevent hyperpigmentation.

A practical approach to the therapy of ocular lesions due to air bags has been proposed by Lee et al.[30] Patients referred for ophthalmologic examination should be submitted to evaluation of visual acuity, ocular mobility, slit lamp examination and pH check of lacrimal fluid. CT scan of the orbits is mandatory in case of altered ocular mobility and severe eyelid and periorbital tissue damages. The usual treatment for corneal abrasions, detected by means of a slit lamp with fluorescein dye, is represented by antibiotic ointments or ophthalmic solutions, cycloplegics, *N*-acetyl-L-cysteine, and lubricants.[34]

Alkaline exposure presents an intrinsic high risk for corneal injury and induces a potential vision-threatening lesion.

An ocular pH of 8 (normal values 7–7.4) requires copious irrigation with saline solution for 1–2 h until pH is stable for 30 min.[34] Conjunctival fornices must be swabbed to remove alkaline deposits that might release alkali remains, which continue to cause damage. Antibiotics and cycloplegics are recommended. Early diagnosis and consequent early treatment are keys to preventing permanent visual impairment.

The knowledge of such a variety of injuries caused by air bags should induce practitioners, emergency doctors, and dermatologists to carry out a careful evaluation of signs and symptoms in patients involved in crashes with air bag activation.

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80 Cigarette Smoking and Skin

Yung-Hian Leow

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80.1 INTRODUCTION

Smoking is a major health concern in all communities in the world. It is associated with various medical morbidities and mortality, namely, pulmonary malignancies, chronic obstructive lung diseases, ischemic heart disease, stroke, and other serious internal diseases. Smith and Fenske[1] highlighted the often neglected skin concern with smoking.

The concern with the skin may be considered as trivial as outlined by Keough[2] in his rather candid editorial on smoking being an ugly habit. Nonetheless, to the ordinary men in the street, it may be the major deterrence to kicking the habit of smoking.

80.2 REVIEW OF CLINICAL EVIDENCE

There had been numerous publications that outlined the deleterious effect of smoking on the cutaneous vasculature and oxygenation of the skin.[3]

Mosely and Finseth[4] observed that the healing process on smokers' hands was poor. The same investigators also demonstrated that systemic administration of nicotine impaired healing in experimental animals.[5] Further work by Lawrence et al.,[6] Craig and Rees,[7] and Nolan et al.[8] demonstrated impaired flap survival in experimental animals that had been exposed to cigarette smoke.

There had also been numerous case reports on human studies. Wilson and Jones[9] reported two men who developed immediate vascular insufficiency following smoking one cigarette on the fifth postoperative day after receiving revascularization surgery on their thumbs. There was definite clinical documentation of viable digits before the patients smoked. Harris et al.[10] also reported two cases with impaired circulation of replanted digits, following smoking in the critical postoperative period, 8 and 48 h, respectively.

Rees et al.[11] reviewed 1186 face-lifts procedure that had been performed over a 6-year period. They found that 10% of cases was complicated by sloughing of the skin. Eighty percent were smokers at the time of the surgery. In a prospective study on 83 patients who underwent rhytidectomies and in a retrospective study on 156 patients who underwent frontal hairline flap reconstructive surgery, Riefkohl et al.[12] and Dardour et al.[13] found positive evidence suggesting an association between smoking and skin necrosis.

Goldminz and Bennett[14] reported a significant dose-response effect between the number of packs of cigarette smoked per day and the development of skin necrosis in 916 flaps and full-thickness grafts that had been performed in 200 patients. Current high-level smokers who smoked one or more packs per day developed necrosis approximately three times more frequently than nonsmokers, low-level smokers (i.e., less than one pack per day), and former smokers, thus affirming the compelling evidence between smoking and skin morbidity. Cigarette smoking is also associated with the negative outcome of human pregnancies, namely, low birth weight, increased perinatal mortality, and placental abnormalities.[15]

These reports suggested that the damaging effect of cigarette smoking was mediated through the vasoconstrictive and hypoxic effect of nicotine, carbon monoxide, or possibly other toxic substances produced by cigarette smoking.

80.3 NONINVASIVE MEASUREMENT OF CUTANEOUS VASCULATURE AND TISSUE OXYGEN

Numerous techniques have been employed to evaluate cutaneous blood flow and tissue oxygenation following smoking. Earlier studies measured decrease in microcirculatory flow indirectly,[16,17] but now it is possible to evaluate cutaneous blood flow and tissue oxygenation directly.

Various bioengineering techniques are well outlined in standard textbook and numerous publications.[18] Techniques include laser Doppler flowmetry (LDF),[19–25] videomicroscopy,[26] thermography,[22,25] pulse plethysmography,[27] calorimetry,[28] multi-lead plethysmography,[29] venous occlusion photoplethysmography,[30] photoplethysmography,[31] pulse oximetry,[31] transcutaneous oxygen electrode,[32] and tonometer with an oxygen electrode.[33]

Table 80.1 summarizes most of the work that had been performed by various investigators of which the most commonly employed technique to study the acute effects of smoking on cutaneous blood flow was LDF.

Basically, the studies uniformly demonstrated decreased cutaneous blood flow in smokers, with or without pharmacological intervention, though there may be variations with the different anatomic sites chosen for specific studies. It was however difficult to demonstrate the possible association between smoking and the reduction in tissue oxygenation, with differing results from different studies.

TABLE 80.1
Summary of Studies

Investigators	Method of Measurement	Study Findings in Smokers
Van Adrichem et al.[19]	LDF	Decreased CBF
Richardson[20]	LDF	Lower reactive hyperemia in smokers
Goodfield et al.[21]	LDF	Decreased CBF
Bornmyr and Svensson[22]	LDF, thermography	Decreased CBF and skin temperature
Tur et al.[23]	LDF	Longer recovery time and lower peak flow with reactive hyperemia
Baab and Oberg[24]	LDF	Increased gingival blood flow, decreased CBF
Lecerof et al.[25]	LDF, venous occlusion plethysmography	Decreased CBF, inhibited by doxazosin
Richardson[26]	Videomicroscopy	Decreased capillary blood flow velocity
Saumet et al.[27]	Pulse plethysmography, calorimeter	Decreased WTG and PWA
Suter et al.[28]	Multilead plethysmography	Decreased CBF at different anatomic sites
Bournameaux et al.[29]	Venous occlusion plethysmography	Decreased digital blood flow, no change in Tc O_2 tension
Netscher et al.[30]	Photoplethysmography, pulse oximetry	Decreased blood flow, no change in O_2 saturation
Ahlsten et al.[31]	Transcutaneous O_2 electrode, oxymonitor	Weaker postischemic hyperemia response in infants of smoking mothers
Jensen et al.[32]	Tonometer, oxygen electrode	Decreased subcutaneous tissue O_2 saturation
Waeber et al.[40]	LDF	Decreased CBF
Nicito-Mauro[42]	LDF	Decreased blood flow

Note: LDF = laser Doppler flowmetry; CBF = cutaneous blood flow; WTG = water thermal gradient; PWA = plethysmographic wave amplitude; Tc = transcutaneous.

80.4 POSSIBLE PATHOGENETIC MECHANISMS

Nicotine is singled out as the major deleterious agent in cigarette smoke. However, there was little evidence-based data to support the postulate that nicotine is the major causative agent for the vasoconstriction and hypoxic effects of cigarette on the skin.

Other neural and humeral factors may be responsible for vasoconstriction and hypoxia associated with cigarette smoking.

80.4.1 Prostaglandins

Nicotine inhibits the release of prostacyclin synthesis through the inhibition of cyclooxygenase.[33] It induces the release of potent vasoconstrictor thromboxane A2.[34] It had previously been demonstrated that there was reduced biosynthesis of prostacyclin in the umbilical arteries of infants born to smoking mothers.[35,36] Goodfield et al.[21] demonstrated that interference of prostaglandin production with aspirin might be responsible for the vasconstrictive effect of smoking. Nadler et al.[37] also reported that there was reduced urinary excretion of prostacyclin metabolite, 6-keto-prostaglandin F1-alpha in smokers who smoked nicotine-containing cigarettes but not with nicotine-free cigarettes.

80.4.2 Vasopressin

Smoking also induces the release of vasopressin that may be responsible for the vasoconstrictive effect on the skin.[38,39] Waeber et al.[40] demonstrated that reduction in cutaneous blood flow could be prevented by pretreating 12 male volunteers with a vasopressin antagonist.

80.4.3 Sympathetic Nervous System

Smoking, as simulated by the infusion of nicotine can activate the sympathetic nervous system that in turn mediates the positive chronotropic and inotropic effects of the cardiovascular system.[41] Lecerof et al.[25] demonstrated that peripheral vasoconstriction that can be caused by smoking could be inhibited with the use of doxazosin, a selective alpha-1 adrenoceptor blocker.

80.4.4 Calcium-Mediated Homeostasis

By employing LDF, Nicito-Mauro[42] demonstrated that vasoconstriction of the posterior tibial artery in 12 elderly habitual smokers could be prevented by pretreating them with either nifedipine, a calcium channel blocker, or calcitonin, a hormone that can induce hypocalcemia. It was postulated that the vasoconstriction induced by smoking is a calcium-mediated process.

80.4.5 Miscellaneous Mechanisms

Other possible pathogenetic mechanisms that had been postulated include an increase in blood viscosity, platelet aggregation, and damage to the endothelium of blood vessels.[43,44]

80.5 CONCLUSION

Noninvasive skin physiology studies, namely, bioengineering techniques have been deployed to demonstrate objectively the effect of cigarette smoking on the cutaneous vasculature. All studies showed that smoking decreases cutaneous blood flow, though there is some controversy as to whether the change is site-specific. Lowering of the tissue oxygenation by smoking could not be confirmed. There is a wide variation in the study designs, study techniques, and the study populations, thus making direct comparison among all published studies difficult.

The direct relationship between blood flow and the "smokers facies" cannot be firmly established. Nonetheless, apart from establishing the diseased microcirculation of smokers, these noninvasive techniques can be effectively used in various fields of medical practice, in particular, wound healing, management of infants born to smoking mothers, smoking cessation program, and in the emerging field of esthetic medicine and appearance-based dermatology.

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81 Chemical Analysis of Tattoo Pigments Cleaved by Laser Light

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81.1 INTRODUCTION

Tattooing, an ancient art traced back to the Stone Age, has remained popular throughout time and across many cultures and continents [1]. The number of tattooed individuals has increased significantly, especially among youth. In the United States ~16% of the population is tattooed, whereas in Europe it is ~10% [2]. Cosmetic tattoos to mimic eye, lip, or eyebrow liner have also become increasingly popular [3].

In the past, coloring agents were inorganic pigments, whereas for dark-blue amateur tattoos, commercially available ink is still in use. Since tattoo compounds in comparison to cosmetics are not officially controlled, the origin and chemical structure of these coloring agents are hardly known. Consequently, neither the tattoo artist nor the tattooed patient has any information about the compounds punctured into skin.

Recently, an extensive analysis of a large number of tattoo compounds was performed for the first time [4]. Most of the commercially available tattoo compounds are organic pigments classified by their chemical constitution [5].

In the past years, adverse reactions have been published in the literature [6–9]. Moreover, several malignant lesions have occurred in tattoos (maybe coincidental) [10–12]. Owing to an improved self-image or social stigmatization, a significant number of people undergo a therapy of tattoo removal by using predominantly Q-switch lasers. The majority of tattoo pigment is found within cells, and not free, within the dermis. While many pigment particles measure "a few microns," others are significantly larger [13] or when accumulated within cells may act as larger aggregate bodies. According to the principles of selective photothermolysis [14], the laser impulses show a high intensity and ultra short pulse durations of a few nanoseconds (Q-switched lasers). The laser pulses change the shape and the size of the tattoo particles abruptly as proven by histology [13].

However, the exact mechanisms of action regarding the destruction of tattoo pigments are still unclear. After being absorbed in the pigment molecule, the energy of the laser light is converted into heat or breaks chemical bonds inside the molecule. The ultra short heating (~ns) of the pigment may lead to disruption of the pigment. At the same time, the extremely hot surface of the pigment raises a rapid expansion of the surrounding water, inducing a negative pressure and a shock wave near to the surface of the pigment. As demonstrated for a suspension of small particles in water, these shock waves may help to destroy the tattooed compounds [15].

As a response, a multitude of mechanisms may occur at the same time. Particles pulverize and form a solution of pigment molecules. Molecules can break up, resulting in decomposition products or molecular structure change. Owing to fragmentation of the tattoo particles, the skin then releases small pigment particles, unknown decomposition products, and newly generated chemical compounds via lymphatic system. All these mechanisms induce a decrease in the color strength of the pigments responsible for a noticeable clearance of a tattoo.

There is no clinical approval of the tattoo pigments punctured into the skin [16,17], and there are no investigations regarding the decomposition products induced by laser therapy of tattoos, so far. In view of the numerous patients treated with those laser systems, it is desired and necessary to investigate the decomposition products of tattoo pigments induced by high laser intensities.

The major goal of the present investigations was the first quantitative analysis of tattoo pigments after laser irradiation by means of high-performance liquid chromatographic (HPLC) and mass spectrometry. For the present investigations, the red pigment "Cardinal Red" (CR) and I8 were used exemplarily. Both are widespread tattoo pigments analyzed previously [4]. It is well known that red pigments cause many

allergic reactions [8] even without laser irradiation. CR and I8 are monoazo pigments, whose azo group cleave either by thermal energy or even in the electronically excited state after light absorption. It is well known that an increase of temperature in azo dyes above 280°C leads to 3,3′-dichlorobenzidine [18], a proven carcinogen of, for example, human lymphocytes [19]. The laser irradiation causes the temperature in such compounds to rise higher than 280°C.

81.2 MATERIAL AND METHODS

The monoazo pigments CR (P.R. 22, Color Index No. C.I.12315) and I8 (P.R. 9, C.I.12460) were purchased from National Tattoo Supply, 485 Business Park Lane, Allentown, Pennsylvania, 18109-9120 [20].

Using a frequency-doubled Nd:YAG laser (Wavelight, Erlangen, Germany) at a wavelength of 532 nm, which is absorbed in CR or I8, the overall volume of the pigment suspensions (2.3 mg in 0.3 ml acetonitrile) were irradiated with light impulses of 15 mJ, a pulse duration of 8 ns, and repetition rate of 10 Hz for 10 min leading to total light dose of 90 J. The spot size was 1 mm yielding a fluence of 2 J/cm² [20].

After laser irradiation the suspension was filtered using PTFE-filter (0.2 µm pore size), and 100 µl diethylenglykoldimethylether (Diglyme) was added. The concentration of the filtered, clear solution was increased up to 100 µl by stirring and flowing nitrogen gas (0.2 bar, 3–4 min). After that, the solution was fed into the modular HPLC system. The system consists of HP1050 Quaternary Pump Mod. Nr. 79852AX, HP1050 Autosampler Mod. Nr. 79855A, HP 4-Channel-Online-Degasser, Mod. Nr. G1303AX, and an Agilent 1100 Photo-Diode-Array-Detector Mod. Nr. G1315b. The analytical column used was a Synergi Max RP 12 (150 × 2.0 mm I.D., 4 µm particle size) from Phenomenex (Aschaffenburg, Germany). Gradient elution was done with water with 0.0059% (w/v) trifluoro acetic acid (solvent A) and acetonitrile (solvent B) at a constant flow rate of 400 µl/min. A gradient profile with the following proportions of solvent B was applied (*t* [min], %B): (0, 10), (40, 48), (60, 98), (70, 98). The compounds described were monitored at 258 nm. The injection volume was 10 µl [20].

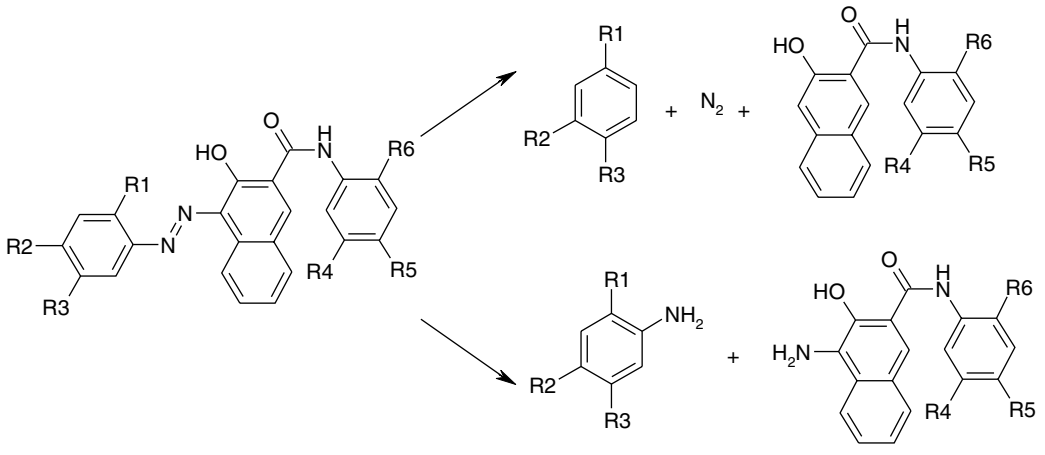
The concentrations of 2-methyl-5-nitroaniline (2-MNA), 4-nitrotoluene (4-NT), 2,5-dichloroaniline (2,5-DCA), and 1,4-dichlorobenzene (1,4-DCB) (Merck KGaA, Germany) in the solutions were determined by the method of internal standard. For each compound, the calibration factor of the compound was determined in a calibration run (single-level calibration). The respective concentration of the standard was chosen to be in the range of the concentration of the decomposition product.

A triple stage mass spectrometer (TSQ 7000, Thermoquest Finnigan, Toronto, Canada) was used to determine the respective mass of the chemical compounds, in particular, the laser-induced products.

To heat up the pigments the suspensions were filled into a glass reaction vessel (supelco micro 33295, Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) and kept in an oil bath at 50, 100, 150, or 200°C for 30 min.

81.3 RESULTS

In Figure 81.1, the decomposition pattern of CR and I8 is shown [20]. The absorbed laser light leads to the cleavage of the azo group of the pigment molecules. As a result



R1	R2	R3	R4	R5	R6	Pigment
Cl	H	Cl	H	H	OCH_3	I8
CH_3	H	NO_2	H	H	H	Cardinal Red

FIGURE 81.1 The chemical structure of P.R. 22 and P.R. 9 used as coloring pigments in Cardinal Red (CR) and I8, respectively. For both pigments, the possible decomposition pattern is shown. Additional change of the decomposition products is possible (chlorine, oxidation). The substituents of the pigment molecules are listed in the table inside the figure [20].

2-MNA, 4-NT, 2,5-DCA, 1,4-DCB, naphthol-AS, or methoxy-naphthol-AS should appear in the suspension. The quantification of these compounds was performed by chromatography before and after laser irradiation. Therefore, the chromatography was calibrated for 2-MNA, 4-NT, 2,5-DCA, and 1,4-DCB.

At first, the concentrations of these decomposition products were determined before irradiation (Figures 81.2a and 81.3a). The concentrations were 1.6 ± 0.3 µg/ml (2-MNA), 1.0 ± 0.2 µg/ml (4-NT), 11.8 ± 0.3 µg/ml (2,5-DCA), whereas the concentration of 1,4-DCB was below the detection limit of the system used. Next, the irradiation of the pure solvent showed no effect.

After the laser irradiation of the tattoo pigments, the concentration of the decomposition products increased significantly (Figures 81.2b and 81.3b). When using CR the concentration of 2-MNA or 4-NT increased 33- or 45-fold, respectively. With I8 the concentration of 2,5-DCA or 1,4-DCB increased 7- or 33-fold, respectively (Table 81.1). Mass spectrometry confirmed the identity of these decomposition compounds.

Additionally, the UV/VIS spectra (data not shown) showed an excellent correlation of the decomposition products

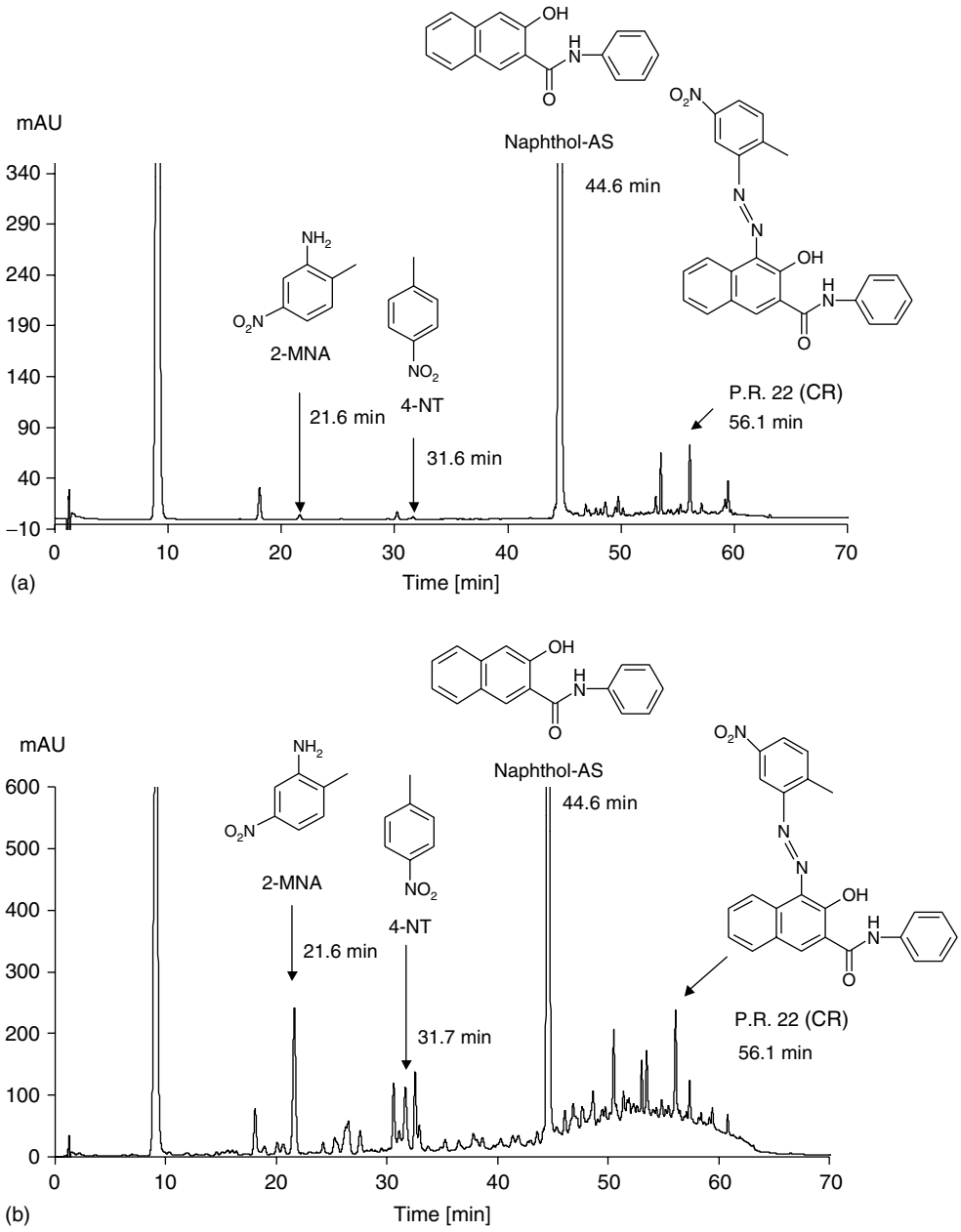


FIGURE 81.2 The chromatogram of Cardinal Red (CR) before (a) and after (b) laser irradiation. Every compound fed into the HPLC needs a certain time (min) to appear at the detector. Every peak corresponds to a different compound. The first peak (without any description) is the tracer used for HPLC. To achieve better illustration of the data, different scales are used for the intensity of HPLC detection (mAU). The chemical formulas of the coloring pigment P.R. 22 and of the decomposition products are included in the diagram such as 2-methyl-5-nitroaniline (2-MNA), 4-nitrotoluene (4-NT), and naphthol-AS [20].

and the respective standards for 2-MNA, 4-NT, 2,5-DCA, 1,4-DCB, and naphthol-AS. Since methoxy-naphthol-AS was not available as pure substance, this compound was identified by mass spectrometry. As shown in Figures 81.2 and 81.3, many other products appear in the suspension after laser irradiation but are unidentified so far.

The peak of CR in Figure 81.2b (after laser irradiation) appears higher than the respective peak in Figure 81.2a. It seems that there is at least one further decomposition product (after laser irradiation) hidden in the CR peak at 56.1 min (Figure 81.2b), which is not obvious at the HPLC wavelength of 258 nm. However, a double peak appeared in the HPLC isoplot at 558 nm, which was not present before laser irradiation (data not shown).

The better solubility of naphthol-AS and methoxy-naphthol-AS in the solvent used leads to HPLC peaks higher

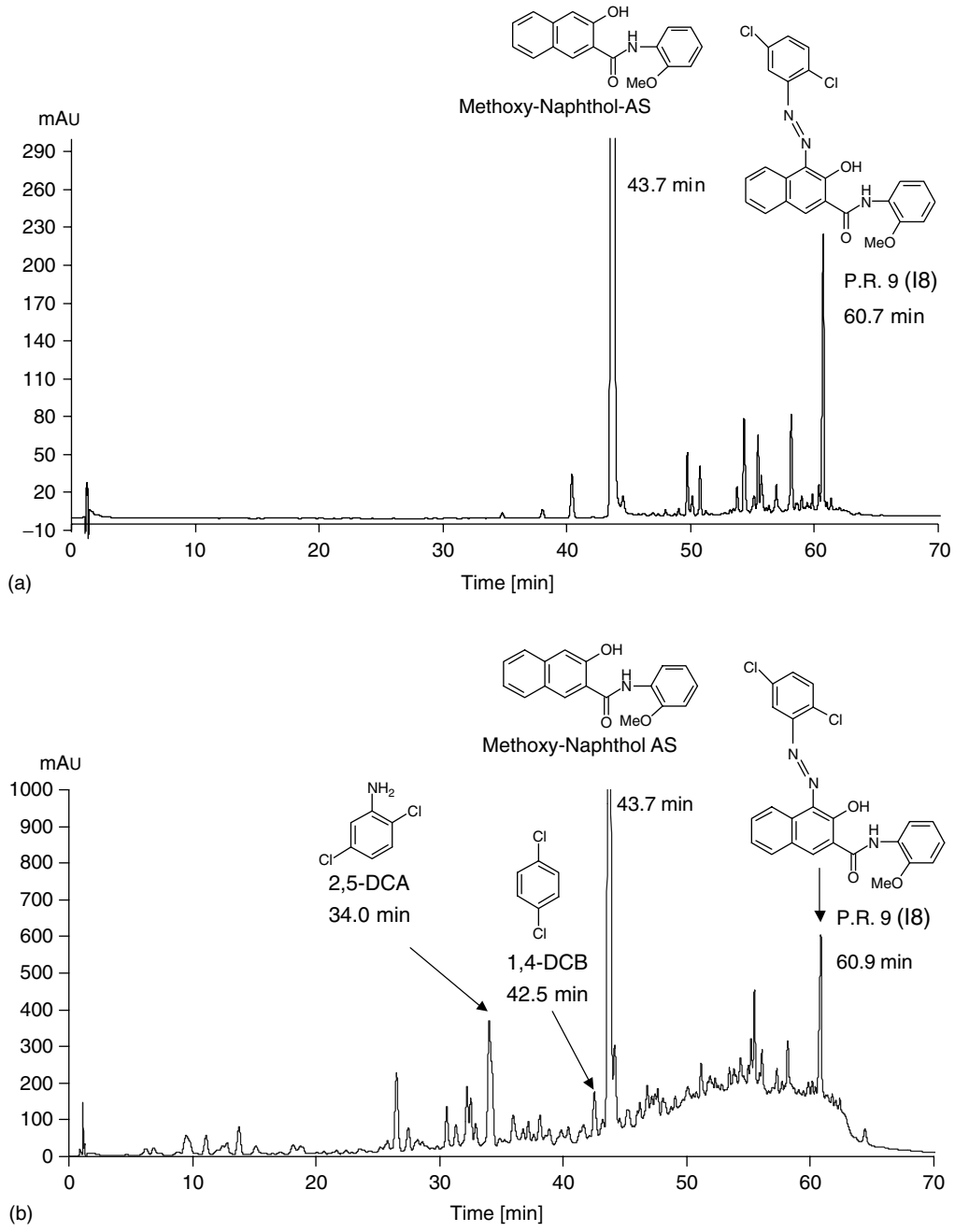


FIGURE 81.3 The chromatogram of I8 before (a) and after (b) laser irradiation. Every compound fed into the HPLC needs a certain time (min) to appear at the detector. Every peak corresponds to a different compound. The first peak (without any description) is the tracer used for HPLC. To achieve better illustration of the data, different scales are used for the intensity of HPLC detection (mAU). The chemical formulas of the coloring pigment P.R. 9 and of the decomposition products are included in the diagram such as 2,5-dichloroaniline (2,5-DCA), 1,4-dichlorobenzene (1,4-DCB), and methoxy-naphthol-AS [20].

TABLE 81.1
The Amounts of Decomposition Products Before and After Laser Irradiation Regarding the Pigments Cardinal Red (CR) or I8

	CR		I8	
	Before Irradiation (g/ml)	After Irradiation (g/ml)	Before Irradiation (g/ml)	After Irradiation (g/ml)
2-MNA	1.6 ± 0.3	53.1 ± 10.1		
4-NT	1.0 ± 0.2	44.7 ± 8.2		
2,5-DCA			11.8 ± 0.3	79,6 ± 1,4
1,4-DCB			< 0.5	32.6 ± 0.4

Note: The products found are 2,5-dichloroaniline (2,5-DCA), 1,4-dichlorobenzene (1,4-DCB), 2-methyl-5-nitroaniline (2-MNA), 4-nitrotoluene (4-NT). Norm is the maximum normalization of the full wavelength range of the spectra shown [20].

as compared to the pigment red or I8 peaks. The wavelength of the laser (532 nm) is well absorbed in the pigments but not in naphthol-AS or methoxy-naphthol-AS.

During laser irradiation, the temperature of the pigment suspensions increased slightly and the pigments might be cleaved by the thermal energy of the suspension to a certain extent. To check possible effects of elevated temperatures, the pigments were heated up to 200°C in a separate study. The suspensions were investigated by chromatography after being heated up to 50, 100, 150, or 200°C without laser irradiation. However, the chromatogram of the heated suspensions remained nearly unchanged.

81.4 DISCUSSION

Many tattooed people decide to remove their tattoos. Besides adverse reactions [6,7,17,21,22] of the tattoo pigments itself, the main reasons for removing tattoos are improved self-image or social stigmatization. Traditional modalities are the removal of the pigment-containing skin using salabrasion [23], cryosurgery [24], surgical excision [25], or CO_2 laser application [26]. However, these methods induce permanent scarring. Tattoo removal using selective photothermolysis [1] has significantly lower risk of scarring [27].

Therefore, the removal of tattoos by laser irradiation is a widespread therapy used by physicians of different fields. Tattoos were treated using different laser systems such as ruby lasers (694 nm), alexandrite lasers (755 nm), or Nd:YAG lasers (532, 1064 nm) at the respective wavelength [1,28,29].

The laser wavelength of 532 nm was used in view of the absorption spectrum determined previously [4]. Additionally, at this wavelength the chromatograms show clear absorption for the pigments but not for the contaminating compounds naphthol-AS or methoxy-naphthol-AS (Figures 81.2a and 81.3a). Regarding 694 or 1064 nm the pigments used show an absorption coefficient close to zero. However, a substantial absorption of light energy in the pigments is necessary. In that case, the corresponding energy is converted predominantly into heat, leading to a substantial increase in the temperature of the molecule and consequently inside the pigment particle. This leads to both, the demolition of the pigment crystals and the chemical change of pigment molecules. It seems to be reasonable that both effects contribute clinically to the fading of the tattoo color after laser treatment. In case of a suboptimal wavelength, hardly any or no fading of the tattoo color should take place.

In spite of the numerous patients treated with lasers by physicians, there are no investigations of the decomposition products of the tattoo pigments. When applying the laser energy to the pigment suspension, the results show cleavage of the tattoo pigments and a significant increase (up to 45-fold) in decomposition products. The products are 2-MNA, 4-NT, 2,5-DCA, 1,4-DCB, and naphthol-AS or 1-amino-naphthol-AS. Therefore, the fluence used for the study is within the range of clinical settings of 2–4 J/cm² [28,30,31].

4-NT is toxic as shown with human lymphocytes [32]. 5-Nitro-*o*-toluidine, which is also designated to 2-MNA, may cause liver dysfunction as shown with workers from a hair dye factory [33]. Additionally, 2-MNA is a carcinogenic substance as shown by Sayama et al. [34] using *Salmonella typhimurium* YG, similar to other di-nitro-toluenes. 1,4-DCB has been reported to cause tumors in kidney of male rats and in liver of male and female mice [35], whereas 2,5-DCA was capable of inducing nephrotoxicity in rats [36]. Naphthol-AS or 1-amino-naphthol-AS leads to skin irritation; the toxicology of these compounds are not completely investigated so far.

The HPLC prior to laser irradiation (Figures 81.2a and 81.3a) shows that the tattoo colorants are already contaminated with a variety of other compounds, among them are the same compounds produced by laser irradiation. These impurities are possibly due to the chemical synthesis of the colorants. One has to take into account that these colorants have been never produced for application on humans, although they are injected into the skin like medical drugs. The chemical industry produces such colorants to stain consumer goods. Therefore the chemical industry does not worry itself around these impurities detected by HPLC in the present investigation.

Moreover, laser irradiation induced many other products as shown by chromatography (Figures 81.2b and 81.3b). These products remained unidentified so far due to the complexity

of chemical reactions induced by the laser light. All these compounds may possibly cause adverse effects on the skin as recently reported with an extensive urticarial and indurated reaction 30 min after laser treatment of a tattoo [37]. It is well known that tattoo pigments are transported via blood vessels or lymphatic system in the human body, for example, to the lymph nodes [38] or even to other organs such as the liver. Similarly, the laser-induced decomposition products could be transported in the body. Unfortunately, there are no investigations regarding the transport of the tattoo pigments and its impurities after tattooing as well as for the decomposition products after laser treatment.

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82 Dermatotoxicology of Specialized Epithelia: Adapting Cutaneous Test Methods to Assess Topical Effects on Vulva

Miranda A. Farage and Howard I. Maibach

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Investigating cutaneous effects is a fundamental step in assessing the safety of topical products. The arsenal for evaluating cutaneous effects includes standardized predictive skin patch tests such as single- and multiple-exposure patch tests for irritation and repeat insult patch tests for contact sensitization. However, standard patch test methodologies, which were designed to assess the skin at exposed or partially occluded areas of the anatomy, may not be ideally suited to assessing topical reactions in specialized epithelia, such as the vulva. The vulva differs substantively from skin at other sites in morphology and regional differentiation,[1] tissue structure,[2,3] blood flow,[4] occlusion,[5] and tissue hydration,[5,6] which may in turn influence its susceptibility to topically applied agents.[7–10] This review compares the characteristics of vulvar epithelia to skin at other sites (Table 82.1) and describes research aimed at adapting and developing cutaneous test methods to assess topical vulvar exposures.

82.1 VULVAR ANATOMY AND REGIONAL DIFFERENCES IN TISSUE STRUCTURE

Figure 82.1 illustrates vulvar anatomical features. The vulva is bordered anteriorly by the mons pubis, a mound of tissue bearing a characteristic triangular conformation of pubic hair; posteriorly by the perineum, which separates the vulva from the anus; and laterally by the labiocrural folds, which separate the vulva from the upper thighs. The labia majora, lobes that lie medial to the labiocrural folds, enclose the thinner labia minora. The labia minora surround the interior portion of the vulva, which comprises the vulvar vestibule and the edge of the hymen at the vaginal orifice (introitus). The urethral orifice lies anterior to the introitus. The labia minora join anteriorly to the urethral orifice to form the preputium clitoridis, a hood of tissue that covers the clitoris. The posterior junction of the labia minora forms the fourchette. The anterior and posterior commissures are located at the junctures of the labia majora anterior to the clitoris and posterior to the fourchette, respectively.

The vulva is derived from two embryonic layers, the ectoderm and the endoderm. As a result, vulvar tissue displays regional differences in morphology and structure. The cutaneous epithelium of the mons pubis, labia majora, and perineum, like skin at other sites, is derived from the embryonic ectoderm. It exhibits a keratinized squamous structure with sweat glands, sebaceous glands, and hair follicles (Figure 82.2a). The thickness and degree of keratinization of vulvar skin decreases in moving inward from the labia majora to the surface of the clitoris and the labia minora. The epithelium of the

TABLE 82.1
Comparison of the Skin and Vulvar Epithelia

		Vulva	
Characteristic	**Exposed Skin**	**Keratinized Epithelium**	**Nonkeratinized Mucosa**
Epithelial structure	Keratinized, squamous epithelium with hair follicles, sweat glands, and sebaceous glands. Regional variations in thickness.	*Mons pubis, labia majora*: Keratinized epithelium with hair follicles, sweat glands, and sebaceous glands.[3] *Outer two-thirds of labia minora*: Thinner, keratinized epithelium lacking hair follicles and sweat glands.[2,3]	*Inner third of the labia minora and vestibule*: Thin, nonkeratinized mucosal epithelium comparable in structure to buccal and vaginal mucosae.[2,11]
Langerhans cell densities	Langerhans cell densities range from 400−1000 cells mm^{-2} skin.[59]	Langerhans cell densities similar to the skin.[12]	No difference in Langerhans cell densities between keratinized and nonkeratinized regions.[12] Menstrual cycle unlikely to have an impact.[13]
Occlusion	Occurs at certain sites, e.g., axilla.	Anatomical and garment-related occlusion.	Anatomical occlusion.
Friction	Varies by anatomical site.	Higher friction coefficient than forearm skin.[21]	Not determined.
Hydration	Varies by anatomical site.	More hydrated than exposed skin, based on trans-epidermal water loss (TEWL).[5,20]	Hydrated by cervicovaginal secretions.
Permeability	A function of skin thickness and concentration of hair follicles, sweat glands, and sebaceous glands.	Greater occlusion and hydration may affect permeability relative to other sites, depending on nature of applied vehicle and penetrants. Seven-fold more permeable to hydrocortisone than forearm skin.[24]	More permeable than keratinized skin; comparable to buccal mucosa.[28,29] Characteristics of tissue structure, lipid profile, thickness, hydration, and occlusion lead to increased permeability.[32,33]

Source: Farage, M. and Maibach, H.I., *Contact Dermatitis*, 51, 201, 2004. With permission.

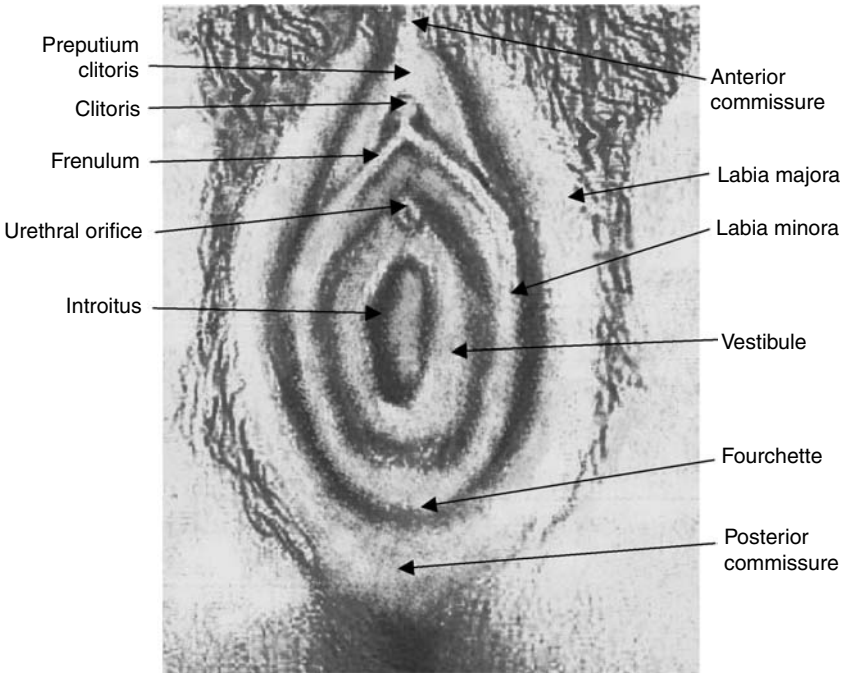


FIGURE 82.1 Anatomy of the vulva. (Farage, M. and Maibach, H.I., *Contact Dermatitis*, 51, 201, 2004. With permission.)

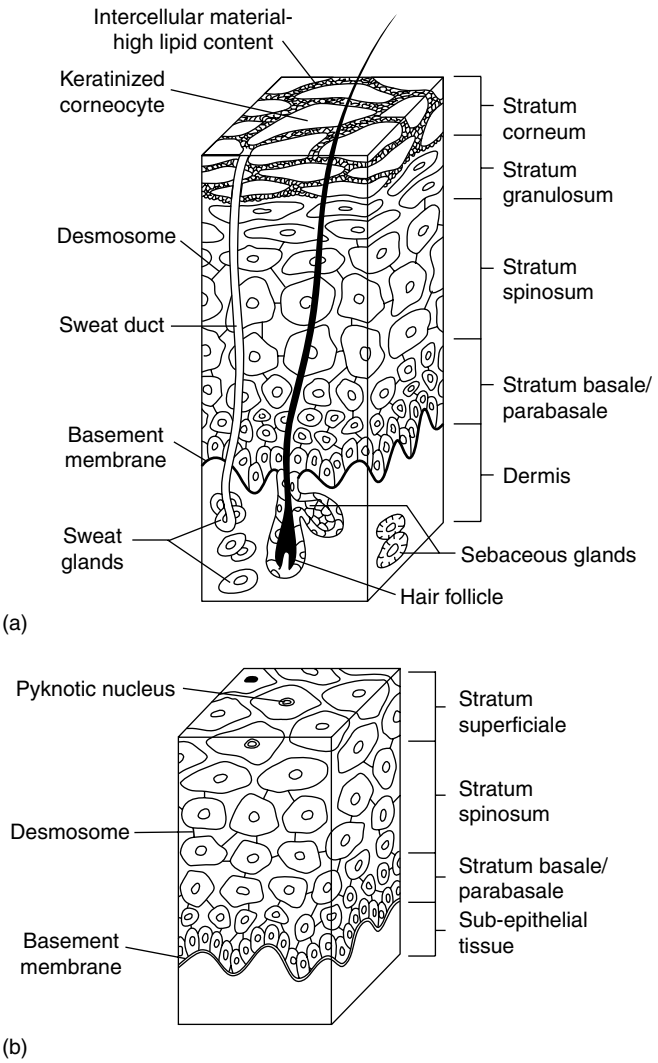


FIGURE 82.2 Structure of vulvar epithelia: (a) keratinized vulvar skin; (b) nonkeratinized vulvar vestibule. (Farage, M. and Maibach, H.I., *Contact Dermatitis*, 51, 201, 2004. With permission.)

labia minora is markedly thinner than that of the labia majora and bears no sweat glands or hair follicles in women of reproductive age.[3]

From approximately the inner third of the labia minora inward to the introitus, the epithelium becomes nonkeratinized (Figure 82.2b). Hart's line, which demarcates the junction of keratinized skin and nonkeratinized tissue, borders the vulvar vestibule. The vulvar vestibule is derived from the embryonic endoderm. Its epithelial structure histologically resembles that of the vagina and the nonkeratinized regions of the oral cavity.[2,11] Its superficial stratum bears large, moderately flattened cells lacking keratin, but containing glycogen granules and frequently pyknotic nuclei. Differentiation of the inner mucosal layers is indistinct: loosely packed, polyhedral cells alter in size and organelle density as they migrate upward from the generative basal layer, but do not form clearly demarcated strata as observed in the skin. Cervicovaginal secretions moisten the vulvar vestibule.

82.2 IMMUNE CELL POPULATIONS AND RESPONSIVENESS

Like the skin at other sites, the vulvar epithelium is an immunocompetent tissue. Langerhans cells are the most common immune cell type in the vulva; intraepithelial and perivascular lymphocytes are infrequently found.[12] Langerhans cells serve as sentinels: they play a role in the induction of allergic contact dermatitis by sampling antigen that crosses the tissue and presenting it to T-cells in the lymph nodes, initiating the hypersensitivity response. No difference in Langerhans cell densities exists between keratinized and nonkeratinized vulvar tissue.[12]

Menstrual cycle variability in vulvar immune cell populations has not been studied directly; however, cyclical variability is not expected because the number and distribution of immune cells in the vagina, a hormonally responsive tissue, remain stable throughout the menstrual cycle. [13]

Although the population densities of resident Langerhans cells are similar in different regions of the vulva, distinct responses to antigen may be possible. Antigen application to vulvar skin can result in sensitization; indeed, allergic contact dermatitis to topical agents is a prime contributor to persistent vulvar discomfort.[14–16] By contrast, antigen application to nonkeratinized mucosa may induce tolerance. This phenomenon, best characterized in the oral mucosa, is not due to the phenotype of resident Langerhans cells, but results from altered responses at the level of the draining lymph nodes.[17–18] The immune responsiveness of the vulvar vestibule has not been studied. However, data from animal models demonstrate that tolerance induction occurs in the histologically similar, nonkeratinized epithelium of the vagina, where the phenomenon is hormonally regulated.[24] In mice, vaginally induced tolerance occurred only during the estrogen-dominant phase of the estrus cycle when sperm exposure would occur. Potentially, therefore, the response to contact sensitizers possibly may differ between keratinized vulvar skin and the nonkeratinized epithelium of the vulvar vestibule.

82.3 BLOOD FLOW, TISSUE HYDRATION, AND OCCLUSION

The vulva differs from skin at other sites in blood flow levels and in the degree of skin hydration and occlusion (Table 82.2). Blood flow in labia majora skin is over twice that in forearm skin.[19] Histamine treatment increases the blood flow in vulvar skin at doses to which forearm skin is unresponsive.[4]

Vulvar skin is more hydrated and has a lower water barrier function than exposed skin. Measurements of trans-epidermal water loss (TEWL) demonstrate that water diffuses across the stratum corneum of the labia majora faster than across the stratum corneum of the forearm (Table 82.2).[5,20] Heightened TEWL results in part from elevated vulvar skin hydration due to occlusion. However, vulvar skin may also present an

TABLE 82.2
Quantitative Comparison of Biophysical Variables, Permeability, and Irritant Susceptibilities in Forearm and Vulvar Skin (Labia Majora)

Parameter Assessed (Units)	Forearm	Vulva	Statistical Significance (n = Number of Subjects)	References
Trans-epidermal water loss (g/m^2·h)	3.5 ± 0.3	14.5 ± 1.3	$p < 0.001$[a] ($n = -44$)	21
Friction coefficient (μ, unitless)	0.48 ± 0.01	0.66 ± 0.03	$p < 0.001$[a] ($n = -44$)	21
Blood flow (absorbance units)	22.0 ± 3.0	59.5 ± 7.4	$p = 0.001$[a] ($n = 9$)	4
Hydrocortisone penetration (% of applied dose absorbed in 24 h)	2.8 ± 2.4	8.1 ± 4.1	$p < 0.01$[b] ($n = 9$)	24
Testosterone penetration (% of applied dose absorbed in 24 h)	20.2 ± 8.1	25.2 ± 6.8	NS[b,c] ($n = 9$)	24
Frequency of irritant reactions to 20% maleic acid solution (%)	62	76	— ($n = 21$)	10
Mean intensity of irritant reactions to 20% maleic acid at 24 h postapplication (0−3 visual scale)	0.86 ± 0.36	1.29 ± 0.83	$p = 0.036$[a] ($n = 21$)	10
Frequency of irritant reactions to 17% benzalkonium chloride solution (%)	9	57	Not determined ($n = 21$)	10
Mean intensity of irritant reactions to 17% benzalkonium chloride solution at 24 h postapplication (0−3 visual scale)	0.19 ± 0.33	1.00 ± 0.88	$p = 0.0003$[a] ($n = 21$)	10
Irritant reactions to 1% sodium lauryl sulfate at day 2 postapplication (proportion of scores > 1 on 0−4 scale)	9/10	0/10	$p < 0.05$[d] ($n = 10$)	9

[a] Student's t-test.
[b] One-way analysis of variance followed by Neuman−Keuls multiple range test.
[c] Not significant.
[d] Wald−Wolfowitz two-sample test.
Source: Farage, M. and Maibach, H.I., *Contact Dermatitis*, 51, 201, 2004. With permission.

intrinsically lower barrier to water loss: steady-state TEWL values remain higher on the labia majora than on the forearm after equilibration with the environment or after the prolonged drying of both sites with a desiccant.[5,6] The comparatively greater hydration of occluded vulvar skin raises its friction coefficient (Table 82.2), which may make vulvar skin more susceptible to mechanical damage.[21]

82.4 PERMEABILITY AND SUSCEPTIBILITY TO IRRITANTS

Predicting tissue permeability is complex. The phenomenon depends on the extent to which the penetrant partitions into the tissue, the rate at which the penetrant diffuses through the tissue, and the distance to be traversed.[22] Consequently, penetration of exogenous agents through vulvar tissue is influenced by regional differences in vulvar epithelial structure, lipid composition, and tissue hydration, as well as the physicochemical characteristics of the penetrants and the nature of the applied vehicle.

82.4.1 Keratinized Labia Majora Skin

The skin of the labia majora exhibits variable permeability to exogenous agents when compared to exposed forearm skin. For example, the skin of the labia majora is substantially more permeable to hydrocortisone than the skin of the forearm (Table 82.2).[23,24] Probable contributing factors include the elevated hydration of vulvar skin relative to forearm skin, the higher concentration of hair follicles and sweat glands, and elevated cutaneous blood flow.

Tissue penetration rates also depend on the properties of the penetrant. For example, there is no difference in the rate of testosterone penetration through vulvar and forearm skin, although the skin at both sites is far more permeable to testosterone than to hydrocortisone (Table 82.2).[24] The rapid skin penetration of testosterone through both the vulva and the forearm may be related to its hydrophobicity as well as to the presence of androgen receptors at both sites.[25]

The keratinized skin of the labia majora also exhibits variable susceptibility to topical irritants. Evidence from

studies on polar irritants suggests that heightened vulvar skin hydration influences this process. Skin penetration of a polar agent depends on its external concentration and its relative solubility in the applied medium and skin tissue.[26,27] Because the stratum corneum is lipophilic, penetration of polar or charged substances is usually disfavored, but hydration of the stratum corneum should facilitate their penetration. Consistent with this hypothesis, concentrated aqueous solutions of the polar irritants, benzalkonium chloride and maleic acid, irritated vulvar skin more than forearm skin.[10]

By contrast, vulvar skin was less affected than forearm skin by the model irritant, sodium lauryl sulfate, applied as a dilute aqueous solution.[2,9] In this instance, one must consider the physicochemical properties of the surfactant as well as its solvation at low concentration by the applied medium. Specifically, surfactants bear both a charged, polar head and a lipophilic tail. Skin penetration of the charged head is disfavored; moreover, in dilute aqueous solution, solvation of the polar head would be difficult to overcome. Consequently, partitioning of the hydrophobic surfactant tail into stratum corneum lipids may have served as the principal driving force for skin penetration, resulting in comparatively higher penetration of the less hydrated forearm skin.

In short, keratinized vulvar skin varies in its susceptibility to topical penetrants when compared to forearm skin. Although the comparative permeability of vulvar skin depends on a combination of factors, in certain instances vulvar skin is more susceptible to topical agents than the skin at other sites. Vulvar skin also has an elevated friction coefficient,[10] which may contribute to breaches in skin integrity. When present, friction and chaffing related to obesity, shear forces associated with impaired mobility, and excess skin hydration due to urinary incontinence may further compromise vulvar skin. Taken together, these considerations support a conservative approach to assessing the potential effects of topical products used on the vulva.

82.4.2 Nonkeratinized Epithelium of the Vulvar Vestibule

Nonkeratinized epithelia generally are more permeable to external penetrants than the skin. The relative permeability of nonkeratinized epithelia has been documented by studies on oral tissue, which, like the vulva, displays regional differences in structure and keratinization. The nonkeratinized buccal mucosa and the thinner nonkeratinized mucosa of the floor of the mouth, respectively, are 10- and 20-fold more permeable to water than keratinized skin.[28] Buccal mucosa is also more permeable than the skin to horseradish peroxidase, although absolute penetration rates of this large molecule are lower than those of water.[29]

The heightened permeability of nonkeratinized tissue results from several factors. First, the absence of a stratum corneum removes a principal barrier to entry of external agents. Second, the more loosely packed cell layers create a structure with less resistance to paracellular movement, the principal route by which most penetrants traverse tissues.[30,31] Third, such tissues have a less structured lipid barrier with lower resistance to molecular diffusion.[32,33] Fourth, thinner epithelia (such as the buccal mucosa and vulvar vestibule) present a shorter path length to be traversed.

Nonkeratinized tissue is also more vulnerable to breaches in tissue integrity, which can augment tissue penetration. For example, buccal tissue was 40-fold more permeable than keratinized skin to the organic base, nicotine, an irritant that increases the penetration of coadministered compounds.[34,35]

The heightened permeability of the vulvar vestibule may be inferred from studies on vaginal and buccal epithelia, which serve as surrogate tissues. Vaginal and buccal epithelia have similar ultrastructural features and lipid composition.[11] Moreover, comparable tissue penetration rates at steady state have been observed in each for a range of model penetrants, including water, estradiol, vasopressin, and low molecular weight dextrans.[36–39] Like these epithelia, the thin, nonkeratinized vulvar vestibule may be more permeable and more vulnerable to topical agents than keratinized skin.

82.5 ADAPTING DERMATOLOGICAL TEST METHODS TO ASSESS TOPICAL EFFECTS ON THE VULVA

As evidenced above, vulvar tissue differs structurally and physiologically from exposed skin and may be more susceptible to the effects of topical agents. This potential for heightened susceptibility demands a more conservative approach to dermatotoxicology and risk assessment. Because it is not practical to conduct routine predictive testing on the vulva, our laboratories are developing new approaches to adapt cutaneous testing and risk assessment methods to topical vulvar exposures. Specifically, cutaneous test methods have been adapted to make them more sensitive and relevant and these modified approaches are coupled to a more conservative risk assessment process. Three areas of investigation are being pursued:

1. Developing new cutaneous tests to assess combined chemical and mechanical irritation. Protocols have been developed to evaluate articles, such as sanitary pads, for which movement and friction may contribute to vulvar effects.
2. Increasing the conservatism of the quantitative risk assessment (QRA) for induction of allergic contact dermatitis and adapting the human repeat insult patch test (HRIPT) to raise its sensitivity. Together, these approaches are intended to increase the margin of safety when the results of cutaneous testing are applied to assessing vulvar contact sensitization risk.
3. Examining alternative protocols in an attempt to increase the sensitivity of cutaneous tests for acute and cumulative skin irritation.

These strategies are described in detail in Sections 82.5.1 through 82.5.3.

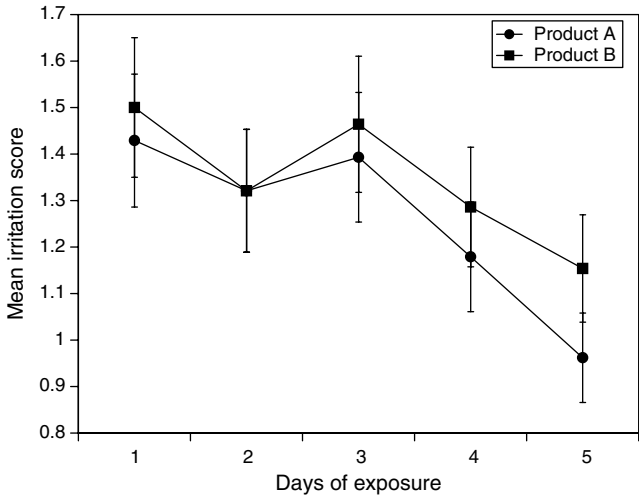


FIGURE 82.3 Comparison of test and control sanitary pads by application to the popliteal fossa ("behind-the-knee" test). (Farage, M., Stadler, A., Elsner, P., Creatsas, G., and Maibach, H., *J. Toxicol. Cutan. Ocul. Toxicol*., 24: 137–146, 2005. With permission.)

82.5.1 Test Methods to Assess Chemical and Mechanical Irritation on Keratinized Skin

A new test method was developed to assess potential mechanical irritation caused by friction by means of repeated topical application to the popliteal fossa (the "behind-the-knee" test).[40,41] The method is principally applied to solid articles, such as sanitary pads, infant diapers, and adult incontinence products, which physically contact with the vulva.

Test materials are applied to the skin of the popliteal fossa, the diamond-shaped area behind the knee joint, for 6 h daily for four consecutive days, and held in place with an elastic bandage. Visual skin grading is performed daily, 30 min after test material removal, using a standard scoring scale for erythema.[42,43] The frequency of subjective reports of sensory irritation is also documented. The test successfully discriminates between materials that are physically irritating due to friction and those that are not.[40] It can be used to test sections of product and can be modified to include wet sample application and testing on compromised skin.

In validation studies, the test successfully discriminated the mechanical irritation potential of three commercially available sanitary pads that were expected to differ in their surface properties.[41] Moreover, the ranking of visual irritation scores associated with each product paralleled the frequency of subjective reports of irritation. Our laboratory is presently using this test for the premarket evaluation of prototype products (Figure 82.3).[44]

82.5.2 Assessment of the Risk of Induction of Allergic Contact Dermatitis

The potentially heightened permeability of vulvar tissue to topically applied agents has direct bearing on the risk of induction of allergic contact dermatitis. In order for the induction of allergic contact dermatitis to occur, externally applied contact allergens must first cross the tissue surface in order for the antigen to become available to resident Langerhans cells. Because most data on contact allergy are derived from exposure to skin at other sites, extrapolating to vulvar exposures requires an extra measure of conservatism to account for tissue permeability differences.

To address this, two approaches have been employed. First, a higher margin of safety has been incorporated into the risk assessment process (QRA) for the induction of allergic contact dermatitis. In brief, QRA is a systematic method for estimating the health risk of chemicals that cause dose-dependent, threshold effects.[45] The process compares the estimated exposure to the potential contact allergen during product use to a safe, reference value[46] derived from an experimentally or clinically determined sensitization induction threshold. To derive the reference value, the experimental threshold dose is divided by sensitization uncertainty factors that account for the need to extrapolate from experimental exposure conditions to the characteristics of actual consumer exposure.[45,47] We proposed the use of uncertainty factors in the range of 1–10 for extrapolating from exposed skin to vulvar skin, and 1–20 for extrapolating from skin to mucosal tissues (Table 82.3). These ranges are greater than those typically applied to exposure at other anatomical sites. The scientific rationale for these ranges is based principally on permeability differences between exposed skin and vulvar tissues, and has been delineated in detail elsewhere.[48]

Secondly, a modified protocol for the HRIPT has been proposed to assess materials that contact the vulva.[49] The HRIPT is a clinical patch for assessing the potential induction of allergic contact dermatitis.[50] This test is not used for hazard assessment, but may be performed after the QRA to further substantiate that the risk of inducing allergic contact dermatitis is negligible. One traditional protocol, optimized for exposure to keratinized skin, employs a 3-week induction phase of nine 24 h applications with 24 h rest periods (48 h on weekends).[51] Between 100 and 200 subjects are typically evaluated. In 1945, Henderson and Riley[52] discussed the predictive power of extrapolating from a small test population to large exposed population on the basis of statistical considerations. Assuming there exists a fraction p in the population who would become sensitized, the probability that one or more of n independent subjects will exhibit a response is given by a binomial distribution:

$$1-(1-p)^n$$

This predicts that if 5% of the population can be sensitized, the probability that at least one subject will respond in a test of 200 people is greater than 99%. The smaller the proportion of potential respondents, the lower the probability of detection, for example, if potential respondents represent 1% of the population, the probability of detection falls to 87%.

Our objective is to increase test sensitivity for extrapolation to mucosal exposures while maintaining reasonable and practical group sizes. Kligman's pioneering studies

TABLE 82.3
Quantitative Risk Assessment Uncertainty Factors for Topical Mucosal Exposures to Potential Contact Allergens

Product Type	Uncertainty Factor	Rationale
Personal hygiene products Conventional sanitary napkins, incontinence pads	1−10	Contact is predominantly with stratified, squamous keratinized epithelium. The default uncertainty factor range (for differences in body site, skin integrity, and occlusion) applies.
Oral Care Products Dentifrice, mouthwash, chewing gum	1−10	Contact is with a mixture of keratinized and nonkeratinized tissue. For many products, rapid dispersion, limited contact time, and salivary dilution occurs that make the lower end of the range more relevant.
Personal hygiene products Tampons, inter-labial pads, etc. Oral care Products Denture adhesives, overnight tooth whiteners, etc.	1−20	Close, occluded contact occurs with nonkeratinized mucosa may occur for extended periods. Nonkeratinized oral and vulvo-vaginal mucosae are similar in structure and more permeable to molecules. The high end of the range may be applicable.

Source: Farage, M.A., Bjerke, D.L., Mahony, C., Blackburn, K.L., and Gerberick, G.F., *Contact Dermatitis*, 49, 140, 2003. With permission.

demonstrated that induction rates are a function of the dose as well as the number, duration, and spacing of exposures.[53] Since it is not always feasible to increase the applied dose (particularly when testing solid articles), the modified HRIPT protocol employs daily 24 h applications, five days per week, during the induction phase. Consequently, the number of applications increases to 15 and the cumulative exposure duration rises by 67% relative to the traditional protocol. This approach should increase the cumulative exposure dose during the induction period in situations where penetration is more rapid, as is expected to occur in mucosal tissue. Another advantage of the proposed protocol is that it incorporates three repetitions of a five-application induction course while maintaining rest periods. Kligman demonstrated that continuous exposure during the induction phase is less effective at induction than allowing rest periods, but that three repetitions of a five-application induction course increased sensitivity to near-threshold concentrations of allergen.[53] Finally, the pattern of consecutive daily exposure in the proposed protocol is more representative of the way consumers use feminine hygiene products. The proposed advantages of this modified protocol are based on theoretical considerations. Comparative studies of the traditional and modified protocols are planned to further validate this approach.

82.5.3 Modified Skin Patch Tests for Acute and Cumulative Chemical Irritation

Skin patch testing on the back or upper arm has been used historically to evaluate potential irritation from raw materials and product formulations. Increasing the sensitivity of skin patch tests for irritation would be a more conservative approach to assessing materials that contact the vulva. However, increasing patch test sensitivity to mild irritants has proved difficult. We first examined four variations of the traditional, 4-day, semiocclusive patch test, that is, combinations of either wet or dry test materials applied to either intact or compromised skin.[54] When inherently mild, commercial sanitary pads were tested in this manner; however, none of the protocol modifications increased test sensitivity. No significant difference in cumulative skin irritation was observed when the same product was tested by each variation; moreover, none of the protocol modifications revealed any significant differences in paired comparisons of products. Hence, little or no enhancement in irritant effects was detected by these methods when materials with inherently low irritation potential were assessed.

As an alternative approach to increasing test sensitivity, we are examining populations that may be inherently more sensitive to irritant effects. Identifying an appropriate target population presents the first challenge. Although 30–40% of people consider their skin to be sensitive,[55,56] objective measures of irritant reactions demonstrate that a fraction of self-declared, sensitive subjects show no increase in objective responses to chemical probes, whereas individuals self-declared as nonsensitive may respond strongly.[57] A preliminary study by our laboratory identified a self-declared, sensitive skin population with a history of dermatologic complaints or adverse reactions to topical products, household products, or clothing; however, recruitment efforts yielded just 15 eligible subjects of 222 respondents.[58] The limiting factor was the participants' willingness to declare their skin to be sensitive: only 7% of respondents did so, despite the fact that 44% had a history of adverse skin reactions to products or clothing. The self-declared, sensitive subjects displayed directionally higher irritant scores to sanitary pads, physiologic saline, and dilute sodium lauryl sulfate than is typical in a standard 4-day patch test, which suggests that this population might indeed be more responsive.[58] Further investigations in a larger population is necessary to confirm the reproducibility of this observation and more research is planned to assess the utility of irritation screening in sensitive subjects when evaluating products intended for vulvar contact. Incorporating use of biophysical methods to assess skin barrier function and blood flow responses may also increase the ability to detect small changes in irritation potential.

82.6 CONCLUSIONS

This review contrasts the characteristics of exposed skin and vulvar tissue, presenting an evidence-based rationale for heightened vulvar susceptibility to topical agents. Factors that may contribute to this elevated susceptibility include the elevated blood flow, skin hydration, and friction coefficient of vulvar skin relative to exposed skin; reduced epithelial thickness and keratinization in moving from the labia majora to the labia minora and vulvar vestibule; and the heightened permeability of nonkeratinized vulvar tissue.[60,61]

Although the potential for elevated vulvar susceptibility to topical agents is not generally recognized, the clinical evidence is suggestive. Epidemiological studies indicate that the contribution of topical medications and personal products to vulvar allergic contact dermatitis is substantial,[16] and a variety of substances have been implicated in vulvar contact sensitization.[15]

The safety assessment of products that contact the vulva should account for its potentially heightened susceptibility to topical agents. Clinical patch tests on exposed skin, part of the standard repertoire for premarket assessment of topical products, may not sufficiently mimic the characteristics of vulvar exposure. Hence, (1) cutaneous test methods are being developed or modified to increase their relevance and sensitivity, and (2) greater conservatism is being employed when extrapolating from the skin to vulvar exposures. To this end, a new protocol involving repeated application to the popliteal fossa ("behind-the-knee" test) has been designed to investigate chemical and mechanical irritation induced by friction; a modified HRIPT protocol has been proposed to assess materials intended for vulvar contact, and the QRA process for assessing the risk of inducing vulvar allergic contact dermatitis has been refined. However, a more sensitive method of evaluating contact irritants is still sought. Modification of the traditional 4-day, cumulative irritation patch test (by employing wet samples or compromised skin sites) failed to enhance test sensitivity to commercial sanitary pads. Patch testing in subjects who show increased susceptibility to chemical and sensory irritation shows some promise as a means increasing test sensitivity. Biophysical measurements also may boost the ability to discriminate between mild irritants. When used judiciously, these combined approaches will augment the degree of conservatism employed when cutaneous tests are used to evaluate materials that contact the vulva.

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83 Anti-Irritants: Myth or Reality? Overview

Christina Ford and Howard I. Maibach

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83.1 INTRODUCTION

Irritant contact dermatitis (ICD), a condition with multifactorial causes, results from acute and chronic exposure to chemicals found in cosmetics, personal care products, drugs, and during occupational exposure [1]. Prognosis for chronic ICD may be poor; the disease results in lost work and causes significant distress. Thus, we sought to identify substances with anti-irritant potential in hopes of improving our understanding and better serving future patients. The concept of anti-irritants is prevalent in Europe and Asia; however, these substances are often considered to be part of a greater marketing ploy rather than a truly scientific approach to reducing ICD. Our overview attempts to add the available science to this concept. Anti-irritants and their potential clinical uses from human, animal, and *in vitro* studies are summarized in Tables 83.1 through 83.3.

83.2 METHOD

We performed a literature search using PubMed, EMBASE, the library at UCSF, and a hand search in an attempt to investigate products that can be considered anti-irritants in either prevention or treatment.

83.3 STUDY SELECTION

Emphasis was placed on studies that included quantitative and qualitative results and that followed evidence-based dermatological guidelines. We defined an anti-irritant as a moiety that either inhibits (prevents) or treats ICD. For the purposes of this review we focused on clinical markers of irritation, that is, edema, erythema, vesiculation, and diminished barrier function, as these are more readily and objectively assessed via visual scoring criteria, transepidermal water loss (TEWL) measurements, and erythema indices.

83.4 RESULTS

83.4.1 Retinoids

Two studies attempted to reduce the irritation potential of retinoid-based products.

TABLE 83.1
Anti-Irritant Substances and Their Potential Clinical Uses (Human)

Product	Potential Benefit	Treatment Type	Study Design	Comments	Reference
Avene medical water	Reduces scaling associated with retinoids	Adjuvant and traditional treatment	Controlled, open-labeled, randomized. 34 patients used retinoic acid alone for 28 days, 35 patients combined retinoic acid and mineral water. Patients instructed to apply mineral water *ad libitum*	No significant reduction of erythema, burn, or itch	[2]
SC-glucan	Effective inhibition of cytokine-mediated inflammatory response to retinoids; decreased erythema and edema	Adjuvant and pretreatment	Controlled, open-labeled	Moderate *in vitro* results tested against human dermal fibroblasts. Good *in vivo* results in both rabbit model and human patch test	[3]
Homeopathic gels (*Urtica urens*, *Apis mellifica*, *Belladonna*, *Pulsatilla*)	Decreased inflammation caused by methyl nicotinate	Pretreatment	Unknown	Methyl nicotinate nonimmunologic contact urticaria is a primarily pharmacological effect; low clinical significance for irritant dermatitis	[20]
Borage oil	Improved pruritis, erythema, vesicualtion, and oozing in atopic patients	Traditional treatment	Unknown	High in gamma-linoleic acid, presumably the active ingredient	[21]
Aloe vera gel	Improved skin barrier function (decreased TEWL) and decreased erythema caused by topical surfactant (SLS)	Adjuvant therapy	Placebo-controlled, randomized	SLS = sodium lauryl sulfate. Dose-dependent results; 100% aloe vera showed most significant results. Composed of multiple ingredients; actives not entirely known	[5]
Cipamfylline (selective phosphodiesterase-4 inhibitor)	Proposed treatment of surfactant-induced (SDS) irritant contact dermatitis via cytokine inhibition	Traditional therapy	Controlled, blind, randomized	No significant reduction in erythema or TEWL compared to placebo or betamethasone	[7]
Strontium nitrate/chloride	Significantly reduced erythema due to aluminum/zirconium salt solution	Adjuvant therapy	Double blind, vehicle-controlled, randomized	Nitrate and chloride showed similar results. Also very effective against sensory irritation	[1]
Cyclic adenosine monophosphate phosphodiesterase inhibitors	Reduced irritation due to 8% Balsam of Peru	Traditional therapy	Unknown	Topical Balsam of Peru causes nonimmunologic urticaria, not irritant contact dermatitis; results may be coincidental	[8]
Corticosteroid (betamethasone-17-valarate, methylprednisolone aceponate)	Decreased TEWL after SLS-induced irritation	Traditional therapy	Randomized, controlled, open	Anti-irritant efficacy noted at the end of treatment trial	[9]
Perfluoropolyethers (oil in water emulsion)	Decreased TEWL and erythema due to SLS, NaOH, and 20% lactic acid	Pretreatment and traditional therapy	Randomized, double blind, controlled	Dose-dependent results with 5% PFPs showing optimal effect. Applicable to ICD conditions caused by occupational surfactant materials	[6]

TABLE 83.1 (Continued)
Anti-Irritant Substances and Their Potential Clinical Uses (Human)

Product	Potential Benefit	Treatment Type	Study Design	Comments	Reference
Glycolic acid (oil in water vehicle)	Reduced irradiation-induced erythema	Traditional therapy	Controlled, open	Area treated for 7 days	[14]
Natural vegetable fats (rape seed and palm)	Decreased irritation due to SLS	Pretreatment	Randomized, controlled	Reduced irritation less than Eucerin or petrolatum	[4]
Lipophilic extracts of *Isatis tinctoria*	Decreased erythema and TEWL due to SLS	Traditional treatment	Controlled, open, randomized	Significant activity against relevant targets of inflammation	[27]

TABLE 83.2
Anti-Irritant Substances and Their Potential Clinical Use (Animal Studies)

Product	Potential Benefit	Treatment Type	Study Design	Comments	Reference
Alchornea cordifolia	Reduced croton oil–induced edema in mice	Traditional therapy	Controlled randomized trial	African plant used traditionally for treatment of bacterial, fungal, parasitic, and inflammatory disorders	[22]
Steroid/NSAID combination: Adexone/Voltaren	Significantly reduced edema, erythema, and inflammatory markers (PGE) due to sulfur mustard in mice	Traditional therapy	Open, controlled	Moderately more effective against second phase of skin injury (extensive epithelial damage with vesiculation and necrosis) than above treatment	[11]
"Triple therapy" with indomethacin, promethazine, and niacinamide [NSAID, antihistamine, vasoconstrictor]	Significantly reduced sulfur mustard induced erythema in hairless guinea pigs	Pretreatment	Open, controlled	Skin injury still occurred; suggested therapy only effective against initial phase of skin irritation	[12]
Methysergide (serotonin antagonist)	Slight reduction in AA-induced ear edema	Traditional therapy (oral)	Open, randomized, controlled		[23]
Lipoxygenase inhibitors (zileuton, MK886)	Potently suppressed arachidonic acid–induced ear edema in mice	Traditional therapy (oral)	Open, randomized, controlled	Antiedema effects reduced by topical application of leukotriene LTC4	[23]
Cyclooxygenase inhibitors (indomethacin, ketoprofen)	Potently suppressed arachidonic acid–induced ear edema in mice	Traditional therapy (oral)	Open, randomized, controlled	Antiedematous effects reduced by concurrent topical application of PG-E2	[23]
Lavender oil	Inhibited mast-cell degranulation in mice, rats	Pretreatment (topical and intradermal)	Unknown	Inhibiting allergic, not irritant reaction, no human correlation yet	[24]
Capsular polysaccharides of cyanobacteria	Anti-inflammatory agents: Inhibited the croton oil–induced edema in male albino mice	Traditional therapy	Controlled	Required 6 h application; not all strains effective; dose-dependent effects	[25]

Alirezai et al. tested the hypothesis that Avene® medical spring water applied *ad libitum* to acne-affected/retinoid-treated areas would reduce the erythema, scaling, sting, and burn associated with retinoid compounds [2]. This was a controlled, open-labeled, randomized study in which patients over the age of 12 with moderate to severe acne were given either Retin-A (Retin-A®, Johnson and Johnson Laboratories, Raritan, New Jersey) treatment alone (34 patients) or retinoic acid and mineral water (35 patients) to spray *ad libitum* (at least four times/day). There was no placebo-controlled group. Patients were assessed at the end of one and four weeks. After 28 days, patients treated with retinoic acid and mineral water showed reduced scaling (46% of patients treated with retinoic acid and mineral water complained of scaling, compared to 79% of patients treated with retinoic acid alone—groups were similar in size). Overall tolerance

TABLE 83.3
Proposed Study Criteria (Ford, Maibach)

Species	Man, animal	[19]
Treatment sequence	Pretreatment versus adjuvant versus posttreatment	
Dosing	Express in mass/area (μg or mg/cm²)	[19]
Anatomic scale	Requires comment on whether the site tested is relevant to its intended clinical use (face, hands, etc.)	[19]
Anti-irritant metrics	Nonlinear: visual grading versus Linear: Bioengineering techniques (TEWL, capacitance, etc.)	[26]
Proposed mechanism(s)	Decreased penetration of irritant Altered biochemistry or metabolism Irritant/anti-irritant bonding "Barrier" or moisturizer function	[19]

of retinoic acid treatment improved with mineral water (37% versus 12% of patients rated their experience as "very good"). The results suggest that mineral water did not alter the therapeutic action of retinoic acid. Rather, those patients treated with both retinoic acid and mineral water showed slight reduction in their overall acne; whether this was due to the combined therapy or increased compliance secondary to decreased unpleasant side effects was not elucidated. One question raised, but not answered, by this study, however, is whether or not the decrease in irritation shown was due to a simple dilution effect or to actual chemical properties of the mineral water itself.

Kim et al. used retinoid-induced irritation to investigate the cytokine mediators involved. Application of retinoids to human epidermal cells increased mRNA expression of the cytokines monocyte chemoattractant protein (MCP-1) and interleukin 8 (IL-8). They then tested various potential anti-irritant substances for their efficacy in inhibiting these cytokines within *in vitro* human fibroblasts and conducted human *in vivo* patch tests (Draize skin irritation test) to test these same substances against retinal-induced irritation. SC-glucan (a soluble biopolymer produced by *Schizophyllum commune*) was effective at reducing retinal-induced irritation in human and rabbit models; *in vitro* it showed a mild inhibition (10.8%) of MCP-1 and IL-8 [3].

83.5 SURFACTANTS

Schliemann-Willers studied the effects of natural fats against sodium laurel sulfate (SLS)–induced irritation in a randomized study of 20 healthy volunteers tested with a repetitive irritation test. Rapeseed and palm fats had a significant beneficial effect against SLS-induced irritation: rapeseed decreased erythema by 2.7 (visual score); palm fats decreased erythema by 2.5, and TEWL by 25.1% compared to control. Both substances, though, offered weaker protection than Eucerin (Beiersdorf, Hamburg, Germany) and petrolatum [4].

Han et al. studied the anti-irritant capacity of aloe vera gel by combining it with varying strengths of SLS in 15 volunteers. SLS was dissolved in distilled water to 1% concentration. Aloe vera gel was diluted in water to 10, 20, 50, and 100%, and then the SLS solution was mixed with each of the aloe solutions in a 1:1 ratio. This mixture was randomly applied to volar forearms and left occluded under Finn chambers (*Epitest*, Helsinki, Finland) and filter paper for 24 h. Both TEWL and erythema index (E-index) decreased significantly with the 100% aloe vera gel–SLS mixture. Note that aloe vera, when tested alone, also significantly decreased TEWL and E-index over the three weeks that patients were followed. These findings bear particular clinical relevance in that aloe vera seems to have long-term protective effects on the skin when used alone and when combined with known irritant products; no pretreatment was necessary to have the desired effect [5]. However, like the retinoid–mineral water study, this study is also limited by the problem of possible dilution effect. Standard deviations or normal distributions were not shown; it is difficult to know if the reduced irritation was due to active ingredients in the aloe vera itself or due to dilution and subsequent reduced percutaneous penetration of the SLS.

83.6 PERFLUOROPOLYETHERS

Schliemann-Willers et al. tested 5% solutions of perfluoropolyethers (PFP) phosphate gel against four standard irritants commonly found in occupational sites: 5% SLS, 0.5% NaOH, 20% lactic acid (all hydrophilic), and undiluted toluene (hydrophobic). Twenty healthy volunteers were pretreated with PFP and then 30 min later the irritant solutions were applied in this randomized, placebo-controlled double blind study. After two weeks they noted a significant dose-related prevention of the experimentally induced occupational ICD [6].

83.7 IMMUNE MEDIATORS

83.7.1 Phosphodiesterase Inhibitors

Kucharekova et al. studied the anti-inflammatory/anti-irritant effects of cipamfylline in 10 subjects. They compared the anti-inflammatory effects of betamethasone with those of cipamphylline (PDE-4 inhibitor) and placebo in this randomized blind study and found that betamethasone alone showed statistically significant reduction in TEWL (approximately 8 g/m²/h) and E-index [7].

Goyarts et al. showed that topical cyclic adenosine monophosphate PDE inhibitors have a moderate anti-inflammatory effect against Balsam of Peru. One major problem with this study is the fact that the irritation induced by Balsam of Peru is not typical irritation, but rather is nonimmunologic contact urticaria [8].

83.7.2 Corticosteroids

Ramsey and colleagues showed a significant decrease in TEWL (10%) and erythema with betamethasone (applied

twice daily for 7 days) when compared to SLS-irritated skin left untreated in 16 volunteers. Similarly, the Berardesca study showed significant decreases in TEWL with the application of methylprednisolone. Neither study, however, listed mass per unit area of corticoid applied [9,10].

83.8 SULFUR MUSTARD

Sulfur mustard causes immediate blistering and affects primarily the skin, eyes, and respiratory system, with cutaneous manifestations occurring in two stages (early and late) that often require prolonged hospitalization and healing times. Two sulfur mustard studies show significant promise in the development of a product useful for lessening sulfur mustard–induced irritation [11,12]. Dachir et al. effectively used a topical steroid/nonsteroidal anti-inflammatory drug (NSAID) combination to reduce edema, blistering, and epithelial damage [11]. Similarly, Yourick and colleagues diminished erythema with combinations of niacinamide and promethazine and niacinamide, promethazine, and indomethacin, although serious skin inflammation and injury still occurred [12]. Currently, however, these studies bear uncertain clinical significance, as experiments have not yet been performed in human volunteers.

83.9 NATURAL PRODUCTS

Levin describes the use of diluted homeopathic gels (made from *Urtica urens*, *Apis mellifica*, *Belladonna*, *Pulsatilla*) as remedies for the inflammation and vasodilatory erythema caused by methyl nicotinate. Also reviewed is the use of two oils, borage and lavender, for the inhibition of atopic symptoms (pruritis, erythema, vesiculation, and oozing) [13]. No mention is made as to how these substances affect similar ICD symptoms. The importance of these findings cannot be completely understood; however, as further examination of drug vehicles, potency and side effect profiles must be performed to truly determine the extent of clinical relevance concerning such "natural therapies."

83.10 MISCELLANEOUS

83.10.1 Glycolic Acid

Perricone and DiNardo studied the anti-inflammatory effects of topical glycolic acid on skin previously irradiated with the minimum erythema dose of UVB. When UVB-burned skin was treated with glycolic acid for 7 days straight, a 16% reduction in irritation could be observed [14].

83.10.2 Strontium Salts

Gary Hahn defined an ideal treatment for sensory irritation, namely, the subjective complaints of burning, itching, tingling, and stinging [1]. The information remains relevant for two reasons: (1) his research identified that strontium salts, either mixed with nitrate or chloride, could act as anti-irritants when used as an adjuvant to aluminum or zirconium applications, especially in terms of reducing erythema; (2) he showed multiple ways in which strontium effectively reduces sensory irritation—perhaps of less interest to the dermatologist—but surely an important patient concern. We hesitate to classify this as an example of "anti-irritation," as the physical injury from the irritant presumably still exists, and a topical anesthetic could be equally effective in blunting the sensory effects. Still, his work may represent a new avenue to follow in the search for effective anti-irritant substances.

83.10.3 Topical Nonsteroidal Anti-Inflammatory Agents

Topical NSAIDs, such as diclofenac, naproxen sodium, are widely used in Europe and Asia as topical anti-inflammatory agents. Their efficacy has been well established for musculoskeletal symptoms. Although many irritant reactions include dermal inflammation, their value as anti-irritants for skin will require further investigation, and they were not included herein as there exist few evidence-based conclusions regarding their dermatologic use. The major exception consists of a body of experiments documenting that NSAIDs inhibit UVB erythema when used prophylactically [15]. Likewise, experiments have been done proving their ability to inhibit nonimmunologic contact dermatitis, but that mechanism does not apply to true ICD [16]. Finally, it bears mentioning that such products require further testing as they may themselves cause irritation in sensitized patients [17].

83.10.4 Calcineurin Inhibitors

The experimental data for tacrolimus and pimecrolimus relate solely to treatment rather than prevention, and is generally specific to psoriasis and atopic dermatitis. Off label use of both drugs has recently been recommended for treatment of allergic contact dermatitis [18].

83.11 CONCLUSIONS

The data on anti-irritants are incomplete, and the studies herein presented prove that much remains to be done to properly identify substances that can be defined as true anti-irritants. There are, however, significant problems with these studies, as mentioned throughout. The mechanism of action of different anti-irritants is inherently useful information in terms of refining future technologies. Some mechanisms are readily understood, for example, barriers that minimize penetration; others are not readily comprehended in spite of decades of study. Petrolatum, for instance, would fit this characterization. From our investigations there appear to be at least two potential mechanisms to inhibiting irritation: (1) inhibition of percutaneous penetration into the epidermis and dermis [19] and (2) altering the biochemistry and metabolism of the irritant compound as it is applied to the skin. Still, what is lacking in the investigation of anti-irritant compounds is a well-controlled randomized and double-blinded study that has sufficient power, adequate number of controls, and that sufficiently tests both sensitivity and specificity of

the anti-irritant to the irritant substance. We propose that the ideal experiment would test sensitivity of the anti-irritant by having two controls—one tested against the anti-irritant and the irritant mixed together, and one testing the anti-irritant as traditional treatment (applied to the skin after irritation developed). The ideal anti-irritants may in fact exist; further investigation, however, remains to be done.

83.12 SUMMARY

Anti-irritants, whether naturally occurring or man-made, are substances that provide a soothing effect to irritated skin and reduce damage by a variety of mechanisms, including reduced absorption and biochemical manipulation of noxious chemicals. These products show promise in reducing irritation caused by acute and chronic exposure to known irritant chemicals; however, the data on anti-irritants are incomplete. Controlled trials of the efficacy of proposed anti-irritants in reducing ICD in human and animal models are reviewed. According to our literature and hand search, anti-irritants seem promising in treating and preventing a variety of ICD conditions, but their true effects remain *sub judice*. Many studies do not allow deduction of clinical effects. Further experimentation must be performed to assess sensitivity and specificity of each anti-irritant to their specific irritant-inducing substance.

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84 Evaluating Mechanical and Chemical Irritation Using the Behind-the-Knee Test: Review

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84.1 INTRODUCTION

The evaluation of the potential irritant effects on skin is an important part of the overall safety assessment for many consumer products. Such an evaluation usually includes some form of in-use clinical or simulated use testing, with third-party evaluation of the skin condition by a trained grader. The nature of the in-use testing that is conducted is often dictated by the product being tested. For example, laundry products are traditionally tested in protocols requiring immersion in solutions of the product, or wear tests of laundered fabrics [1,2]. Personal cleansing products and baby wipes are tested using forearm wash or wipe tests [3–6]. Catamenial (feminine protection) products are typically tested in in-use clinical studies in which volunteer panelists use the product in place of their normal product [7].

Unfortunately, while the data generated in the in-use clinical studies have been valuable in completing safety assessments, the planning and conduct of such studies presents difficulties. For example, in clinical studies on catamenial products, large panel sizes of at least 30 women per test group must be used to detect differences in skin effects. Since the tests are often designed so that start dates coincide with the panelists' menstrual cycles, results may not be available for a minimum of 4–5 weeks from study initiation. Grading is done by visual assessment of the genitalia and is, therefore, intrusive for the panelists. Each panelist can test only one product at any one time, making side-by-side comparisons more difficult. The in-use test results can be confounded by changing conditions in the vulvar and vaginal regions due to microbiological and hormonal differences throughout the menstrual cycle. Panelists may have a broad range of pad wearing and hygiene habits. Finally, investigations into some areas, such as testing on compromised skin, are not possible due to the nature of the in-use test.

The high cost, slow turnaround time, and possible confounding factors associated with in-use clinical testing for catamenial products result in slow and expensive skin safety programs on these materials. In addition, a reliance on clinical testing presents a barrier to the rapid development of new products.

Standard patch testing has been used as an alternative to in-use clinical testing in the past for early evaluations of skin safety during the product development process. However, patch testing evaluates only the inherent chemical irritation caused by a material and is, therefore, incomplete. Patch testing does not evaluate the potential mechanical irritation component, that is, the potential irritation caused by friction, that is so important for some product categories. Evaluating mechanical irritation for catamenial products is

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particularly important since vulvar skin has been demonstrated to have a higher coefficient of friction than other body sites [8].

We developed an alternative test method for evaluating skin effects that would eliminate some of the difficulties of the in-use clinical test system while still providing reliable results on the potential irritant effects. The resulting test system is the "behind-the-knee" test method (or BTK test), using the popliteal fossa as a test site. In this test protocol, samples are applied to the back of the knee using an elastic knee band. As the panelists go about their everyday activities, normal movements generate friction between the test sample and the skin at the test site, thereby adding the element of mechanical irritation. Thus, the BTK test protocol evaluates the inherent chemical irritation potential and the potential for mechanical irritation [9–12].

Here, examples of results from BTK tests conducted on a variety of materials are presented. Where possible, direct comparisons have been made to the results of in-use clinical testing conducted on the same materials. The results demonstrate that the BTK test is reproducible, giving consistent and reliable results when the same materials are tested repeatedly. The test is capable of detecting subtle differences between very similar products that are consistent with clinical testing and other evaluations.

This is a versatile test system capable of providing meaningful results on a variety of different types of materials. In addition, the BTK test provides results consistent with the results of in-use clinical testing conducted on the same materials, and additional data from over 20 years of in-use clinical tests [13]. Unlike the in-use clinical test method, two products can be tested on the same panelist at the same point in time. In fact, by using a common control material and concurrent panels, multiple products can be compared. Investigative programs are possible since the BTK test is easier to conduct for both the investigators and the panelists, providing results in a shorter period of time at a greatly reduced cost.

Although the BTK test was developed using catamenial products, the test system provides a valuable alternative for evaluating the skin effects of any material where mechanical irritation may play a role in overall skin irritation and consumer satisfaction. It has potential applicability for evaluating textiles, facial tissues, baby and adult diapers, and laundry products that may leave residues on fabrics.

84.2 METHODOLOGY

84.2.1 Basic BTK Test Protocol

The BTK test protocols were modifications of those described previously [9–12]. Test material was placed horizontally and held in place on the popliteal fossa by an elastic knee band of the appropriate size, as shown in Figure 84.1. Menstrual pads, pantiliners, topsheets, and fabrics were tested as is. Tampons are marketed in a highly compressed, cylindrical configuration that would make poor contact with the skin in the BTK test. Therefore, these products were tested as uncompressed samples.

Test materials were removed by the panelists 30–60 min prior to returning to the laboratory for grading and reapplication of test materials. Exposures varied from 24 h/day for 3–5 consecutive days in early experiments to 6 h/day for 4 or 5 consecutive days in more recent experiments.

Visual grading of the BTK test sites was conducted by an expert grader under a 100 W incandescent daylight blue bulb. Scoring was done using a previously described scale of "0" to "4," where "0" is no apparent cutaneous involvement and "4" is moderate-to-severe, spreading erythema or edema [10]. The same grader was used throughout an experiment, and the grader was not aware of the treatment assignments.

In the BTK test protocol, the integrity of the skin can be compromised by tape stripping using Blenderm® tape (3M, St Paul, MN) prior to the first application. In some experiments, this was done by repeatedly applying tape to the area up to 20 times, or until the skin exhibited an erythema score of 1.0–1.5 as per the grading scale described in the previous paragraph.

Study participants were healthy adult volunteers who had signed an informed consent with no medical or skin condition likely to interfere with the test. Unlike the clinical studies, both male and female panelists can be recruited for the BTK studies. Participants filled out a brief questionnaire each day to record any discomfort (itching, chafing, burning, etc.).

84.2.2 Materials Tested

Test materials included fabric, menstrual pads and pantiliners, topsheets from pads and pantiliners, products with and without lotion coatings, tampons, and interlabial pads. A summary of the materials tested and the corresponding sample codes is provided in Table 84.1.

84.2.3 Standard Patch Test and Clinical Methodology

Results of the BTK test were compared to those from standard patch testing or clinical testing protocols. Standard patch testing was conducted as previously described [12]. Briefly, patch sites for test materials and conditions were randomized, and test samples were applied via an occlusive patch. Sites were marked with 0.5% gentian violet to aid in visual grading and to ensure that the patches were applied to identical sites each day for the duration of the test. Test materials were removed by the panelists 24 h after application, and subjects returned to the laboratory for grading and reapplication of test materials 30–60 min later. Test sites were scored in a manner identical to the popliteal fossa test sites, as described in Section 84.2.1.

Study designs for the in-use clinical studies are provided in Table 84.2. In the clinical studies on menstrual pads (samples AGT, M, NL, and N), panelists were randomly assigned one of the two test products. They were provided with product and asked to use it during one or two menstrual periods in

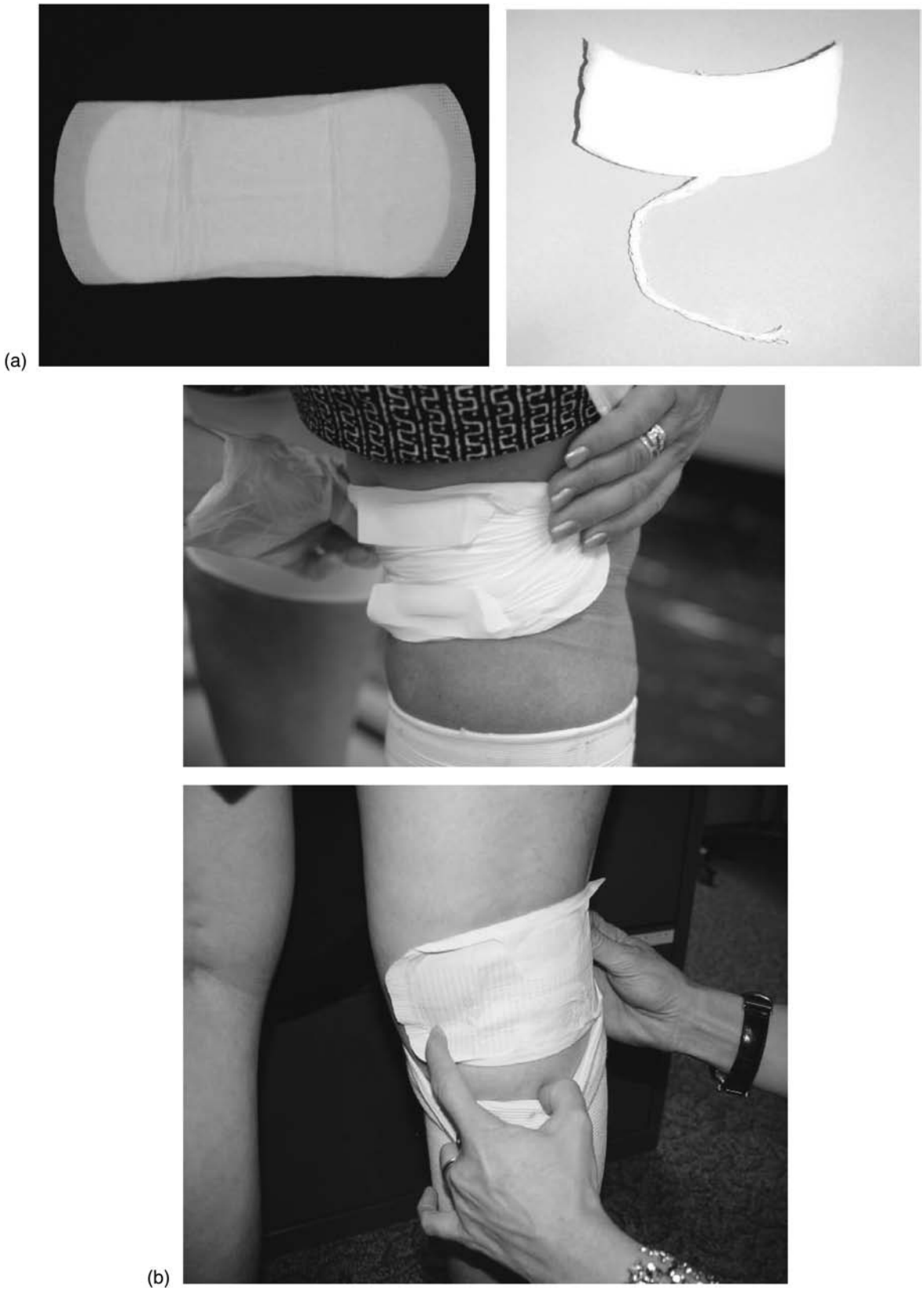


FIGURE 84.1 Test sample application in the BTK test. Testing can be done on a variety of materials, including menstrual pads and uncompressed tampons (a). Test materials are placed horizontally on the popliteal fossa, and held in place by an elastic knee band of the appropriate size (b).

place of their normal product. For the study on the interlabial pads, panelists in the test group were asked to use the test product (sample IL2) for a minimum period of 8 h daily for an entire menstrual cycle (approximately 1 month). A control menstrual pad (A) was to be worn in addition to the test product during menses. The control group used a currently marketed pantiliner (sample P) in place of the interlabial test product. The tampon studies (samples T, U, S, and R) were a crossover design. Half the panelists used one tampon for one menstrual period, then switched to the other for their next period. The other half of the panelists used the tampons in reverse order.

In the in-use clinical studies on menstrual pads, skin condition was assessed by visual grading of the external genitalia for evidence of irritation based on the following scale: 0 = normal skin, 1 = slight erythema, 2 = moderate erythema, 3 = severe erythema, 4 = edema/induration, 5 = skin fissuring, 6 = spreading reaction, and 7 = vesicles/bullae. This grading scale has been previously described in detail [7]. Seven sites are graded separately: mons pubis, labia majora, labia minora, introitus, vestibule, perineal body, and upper thighs. In some studies the clitoris and buttocks were also evaluated.

In the in-use clinical studies on tampons, erythema was evaluated via colposcopic examination based on the following scale: 0 = none, 1 = faint, 2 = moderate, 3 = moderate-to-severe, and 4 = severe. Six sites are graded separately: labia minora, introitus, lower and middle vaginal walls, upper vagina, and cervix.

TABLE 84.1
Summary of Materials Tested in BTK Test

Type of Sample	Number of Times Tested	Test Product Sample Codes
Fabrics	9	Burlap, Satin
Menstrual pads	27	A, B, C, E, H, N, M, AGT
Ultrathin pads	12	AU, BU, TU
Topsheets	12	AT, BT, GT
Lotion-coated samples	7	NL, AUL, ATL, BTL
Tampons	4	T, U, R, S
Pantiliners	1	P
Interlabial pad	1	IL2, IL25

84.2.4 Analyses of Data

In the BTK studies, paired comparisons were conducted using a Wilcoxon's signed ranks test on the irritation scores collected after completion of all test sample applications, unless otherwise stated in the legends of the appropriate tables [14]. In the in-use clinical studies, if the data were normally distributed, evaluation was based on a paired *t*-test. If data were not normally distributed, evaluation was based on a signed rank sum test [15–17].

84.3 RESULTS

84.3.1 Reproducibility and Versatility of the BTK Test

The method has been used in repeated studies on numerous materials, and comparisons between the same materials provide consistent results using a variety of exposure regimens and other protocol variations. Table 84.3 summarizes the results of multiple experiments comparing two control materials for mechanical irritation (burlap and satin) using

TABLE 84.2
Summary of Test Designs for In-Use Clinical Studies

Test Samples					
Code	Description	Code	Description	Study Design	Evaluation Period
AGT:	Menstrual pad with hydrofilm-type polyethylene topsheet.	M:	Menstrual pad with nonwoven topsheet.	Side-by-side comparison	Panelists used same test product used for two menstrual cycles.
NL:	Test menstrual pad with lotion and perfume.	N:	Test menstrual pad without perfume.	Side-by-side comparison	Panelists used one test product for one menstrual cycle.
IL2:	Interlabial pad composed of cotton/rayon core with rayon topsheet.	P:	Nonwinged, regular length, unscented pantiliner composed of pulp absorbant core with polyethylene.	Side-by-side comparison	Panelists used one test product for one menstrual cycle.
T:	Super plus absorbancy tampon (absorbs 12–15 g) composed of rayon with a polyethylene/ polypropylene bi-component outer layer.	U:	Ultra absorbency tampon (absorbs 15–18 g) composed of rayon with a polyethylene/polypropylene bi-component outer layer.	Crossover design	Panelists used one test product for one menstrual cycle, then switched to other test product for a second cycle.
S:	Tampon composed of cotton and rayon absorbant fiber with rayon/polyester outer layer.	R:	Tampon composed of cotton and rayon absorbant fiber with rayon outer layer.	Crossover design	Panelists used one test product for one menstrual cycle, then switched to other test product for a second cycle.

TABLE 84.3
Similar Results with Different Exposure Regimens and Protocol Variations in the BTK Protocol

Exposure Regimen	No. of Panelists	Protocol Variation(s)	Mean Erythema Score ± SE		Significance (p value) (%)
			Burlap Fabric	Satin Fabric	
6 h/day for 4 days	12	None	1.5 ± 0.17	0.55 ± 0.08	<0.02
6 h/day for 4 days	9	None	1.8 ± 0.12	0.38 ± 0.12	<1
24 h/day for 3 days	10	None	1.7 ± 0.15	0.60 ± 0.22	<1
24 h/day for 4 days	13	None	1.9 ± 0.08	0.77 ± 0.26	<1
			Menstrual Pad A	Menstrual Pad B	
6 h/day for 4 days	17	None	1.7 ± 0.06	1.1 ± 0.10	<0.02
6 h/day for 5 days	16	Compromised skin	2.0 ± 0.14	1.4 ± 0.11	<5
6 h/day for 5 days	18	Wet sample	1.6 ± 0.10	1.3 ± 0.09	<5
6 h/day for 5 days	17	Compromised skin and wet sample	1.8 ± 0.11	1.3 ± 0.11	<1

Note: Mean scores for erythema (±SE) at completion of the study were determined. Comparison of mean scores was done using Wilcoxon's signed rank test.

different durations of exposure. Regardless of the specific exposure regimen, that is, 6 h/day for 4 days, or 24 h/day for 3 or 4 days, the burlap produced a significantly higher level of irritation than satin, based on the mean erythema scores.

Repeated studies were also conducted on two menstrual pads with different topsheets: pads A and B. These products have been used repeatedly as controls and standards in the in-use clinical studies, and in other studies for irritation such as the standard patch tests. Both the products are mild to skin in both clinical and patch testing, and during normal use by panelists. However, panelists consistently prefer pad B based on a more pleasing feel and texture (data not shown). Table 84.3 summarizes comparisons of these two samples using 6 h exposures under four exposure conditions that simulate some of the physiological conditions that may occur during normal product use. The exposure conditions included the standard protocol (i.e., dry samples on intact skin), and three protocol variations: dry samples on compromised (tape stripped) skin, wet samples on intact skin, and wet samples on tape stripped skin. In all cases, pad A yielded higher mean scores for erythema than pad B.

Table 84.4 shows results on materials and products where alternative data enabled us to predict the likely results. In the top four examples (Table 84.4a), in-use clinical studies were conducted on the identical materials. In these studies, there were no significant differences in the level of irritation between the products. BTK tests on the same materials yielded the same results as the in-use clinical tests. In the remaining examples (Table 84.4b), the likely results could be predicted based on close similarities between the test materials and previous consumer evaluations, as indicated in the table. In all cases, the BTK test yielded the expected result.

84.3.2 Ability to Test Both Mechanical and Chemical Irritation

In some studies, standard patch tests were applied to the same panelists participating in the BTK test using identical protocol modifications, that is, wet samples and skin compromised by tape stripping. We compared the level of irritation in the BTK test to that shown in the standard patch test to develop a qualitative understanding around the proportion of the overall irritation that could be attributed to mechanical irritation versus chemical irritation. In the examples shown in Figure 84.2, the mean erythema score for the standard patch test is represented as a percentage of the mean score for the BTK. With one exception, the standard patch test produced overall mean irritation results that were 65–85% of the BTK method. The higher overall mean scores produced by the BTK method likely represent the mechanical irritation component of the reaction.

84.3.3 Ability to Compare Several Products Tested Concurrently

In some circumstances, it is desirable to compare the potential irritant effects of more than two products. Table 84.5 shows the result of an experiment in which three panels were run concurrently enabling comparison of the effects of four products. In this study, an experimental topsheet material (topsheet GT) was compared to three menstrual pads (M, E, and H) using three concurrent panels. The topsheet material was similar in irritation potential to all three pads. Since the three panels were run concurrently, and one sample was common to all three panels (i.e., topsheet GT), it was possible to statistically compare the three pads to each other. As shown in Table 84.5, when this statistical comparison was done, menstrual pads M, E, and H were similar in irritation potential.

TABLE 84.4
BTK Test Results on Diverse Product and Material Types

a. Comparison of BTK Test Results with In-Use Clinical Results

	In-Use Clinical					BTK Test[a]			
Materials Tested	**No. of Panelists**		**Results (Mean Erythema ± SE)**		**Significance**	**No. of Panelists**		**Results (Mean Erythema ± SE)**	**Significance**
Pads AGT versus M			Labia majora	Labia minora					
	55	AGT:	0.22 ± 0.04	0.01 ± 0.01	NS	42	AGT:	0.96 ± 0.13	NS
	60	M:	0.20 ± 0.03	0.0 ± 0.0			M:	1.15 ± 0.13	
Pad N versus			Labia majora	Labia minora					
lotioned pad NL	59	N:	0.22 ± 0.05	0.06 ± 0.03	NS	41	N:	2.0 ± 0.07	NS
	60	NL:	0.10 ± 0.04	0.02 ± 0.02			NL:	2.0 ± 0.05	
Interlabial pad IL2			Labia majora	Labia minora					
versus *pantiliner* P	29	IL2:	0.97 ± 0.11	1.24 ± 0.13	NS	22	IL2:	1.4 ± 0.09	NS
	36	P:	0.83 ± 0.12	1.14 ± 0.12			P:	1.4 ± 0.11	
Tampons T versus U			Lower vaginal wall	Middle vaginal wall					NS
	43	T:	0.20 ± 0.04	0.17 ± 0.04	NS	17	T:	1.3 ± 0.09	
		U:	0.08 ± 0.03	0.10 ± 0.03			U:	1.2 ± 0.08	
Tampons R versus S			Lower vaginal Wall	Middle vaginal wall					NS
	67	R:	0.52 ± 0.04	0.38 ± 0.04	NS	15	R:	0.9 ± 0.09	
		S:	0.49 ± 0.04	0.45 ± 0.04			S:	1.0 ± 0.10	

b. Comparison of BTK Test Results with Expected Results

			BTK Test Result			
Materials Tested	**Expected Result**	**No. of Panelists**	**Results (Mean Erythema ± SE)**		**Significance (*p* value) (%)**	**Conclusion**
Pads A versus B	In previous consumer evaluations, pad B was preferred over pad A.	17	A:	1.7 ± 0.06	<5	Pad A gave a significantly higher mean score than pad B.
			B:	1.1 ± 0.09		
Topsheets AT versus BT	In previous consumer evaluations, topsheet BT was preferred over topsheet AT.	11	AT:	1.3 ± 0.25	<5	Topsheet AT gave a significantly higher mean score than topsheet BT.
			BT:	0.59 ± 0.16		
Fabrics: Burlap versus Satin	On the basis of the characteristics of the fabrics, burlap was expected to give a significantly higher score than satin.	12	Burlap:	1.5 ± 0.17	<0.02	Burlap gave a significantly higher mean score than satin.
			Satin:	0.6 ± 0.08		

Note: In-use clinical and BTK studies were conducted as described in the Materials and Methods section. For the BTK studies on standard pads AGT versus M, A versus B, and tampons T versus U, scores were compared using the stratified Cochran–Mantel–Haentzel (CMH) test. For the BTK studies on pads N versus NL, interlabial pad IL2 versus pantiliner P, topsheets AT versus BT, and burlap versus satin, scores were compared using Wilcoxon's signed rank test. For the BTK study on tampons R versus S, scores were compared using analysis of variance (ANOVA). NS = Not significant.

[a] Each 6 h exposure consisted of fresh lotioned samples applied at time 0 and at 3 h.

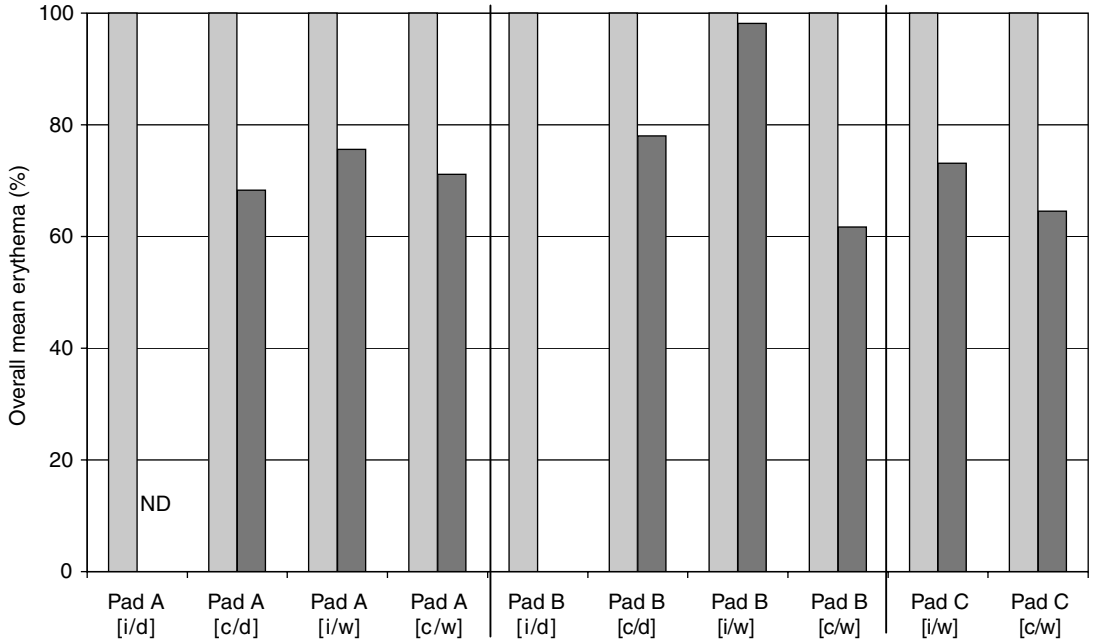


FIGURE 84.2 Representation of the relative contribution of mechanical and chemical irritation. For menstrual pads A, B, and C, the overall mean erythema scores from the standard patch test are expressed as a percentage of the overall mean erythema scores from the BTK test. The data from the popliteal fossa and upper arm test sites were generated in parallel using the same panelists. These studies have been described in detail in earlier publications [9–11]. ND = Not done; i/d = intact skin, dry sample; c/d = tape stripped, compromised skin, dry sample; i/w = intact skin, wet sample; c/w = tape stripped, compromised skin, wet sample; ■ BTK test result (mechanical and chemical irritation); and ■ Patch test (chemical irritation only).

TABLE 84.5
Using a Common Material as a Basis to Compare Multiple Samples

		Number of Panelists	Material Tested	Mean ± SE	Material Tested	Mean ± SE	Significance
Intratrial comparison	Group a	13	Topsheet GT	0.96 ± 0.13	Pad M	1.2 ± 0.13	NS
	Group b	13	Topsheet GT	1.1 ± 0.17	Pad E	1.1 ± 0.16	NS
	Group c	14	Topsheet GT	1.0 ± 0.12	Pad H	0.96 ± 0.08	NS
Intertrial comparison	Groups a and b	13 and 13	Pad M	1.2 ± 0.13	Pad E	1.1 ± 0.16	NS
	Groups b and c	13 and 14	Pad E	1.1 ± 0.16	Pad H	0.96 ± 0.08	NS
	Groups c and a	14 and 13	Pad H	0.96 ± 0.08	Pad M	1.2 ± 0.13	NS

Note: In this study, panelists wore the test materials for 6 h/day for 5 days. An experimental topsheet material (topsheet GT) was compared to three menstrual pads (M, E, and H) using three separate groups of panelists tested concurrently (Groups a, b, and c shown as "intratrial comparison"). Since the topsheet material was a common leg in all the three separate groups, the other three samples (pads M, E, and H) could be compared statistically for differences in irritation reactions (intertrial comparison). Comparison of the final day scores is given. The intratrial comparisons were done using analysis of variance or analysis of covariance (ANOVA/ANCOVA). The intertrial comparison was done using Cochran–Mantel–Haentzel (CMH). NS = Not significant.

84.4 DISCUSSION

An array of materials can be tested for a combination of mechanical and chemical irritation properties in the BTK test (Table 84.1). We have successfully tested menstrual pads, topsheets, tampons, and fabric. The ability to test fabric samples indicates that the test protocol may have applicability for evaluating the properties of fabric materials themselves, or for products and chemicals that may deposit on fabrics, such as fabric softeners and detergents.

The results presented in Table 84.3 illustrate the reproducibility of the BTK test. Comparisons between the same two materials in repeated studies using different exposure regimens and protocol variations produced consistent results. In early development studies, satin and burlap were used as control materials. It was expected that these two

materials would differ markedly in the ability to cause mechanical irritation from friction. As expected, burlap produced a significantly higher level of irritation than satin, whether the exposure was for 6 h/day or 24 h/day. We have also obtained consistent results in repeated tests using other materials and different exposure regimens (data not shown). Comments from participating panelists indicated that the longer exposure time of 24 h became uncomfortable for some panelists, with no real benefit to the quality of the results. Therefore, the current standard protocol calls for 6 h exposure times. However, longer exposures can be used successfully in investigative studies.

Table 84.3 also demonstrates the ability of the test to detect subtle differences between two very similar products: menstrual pads (A and B). Both products are mild to skin and considered nonirritating. However, the BTK test consistently demonstrated that pad A yielded higher mean erythema scores than pad B, regardless of the specific test conditions. This is consistent with the results of consumer and panelist evaluations that indicate panelists prefer pad B over pad A due to a more pleasing feel and texture (data not shown).

The preference for pad B on the basis of the sensations caused by the products (i.e., sensory effects) has been studied in the course of BTK testing [11]. In some studies, panelists kept a daily diary of skin problems experienced at the test sites. Panelists were asked whether they experienced specific sensations such as the sample rubbing against the skin or sticking to the skin, chafing, burning, itching, pain, or any other discomfort. At several scoring time points, the percentage of subjects complaining of the sensations of burning, sticking, or pain was significantly lower for standard pad B when compared to standard pad A.

Table 84.3 also illustrates the ability to incorporate different test conditions in the BTK test. In addition to testing products as is, we tested samples under varying physiological conditions known to occur during use of catamenial products, including conditions where the test materials were wet or the skin may have been compromised. Even under conditions that represent the most extreme physiological conditions of product use, that is, wet product on compromised skin, the BTK test produces reliable results. Such investigations are not always possible in in-use clinical studies, where the specific test site and ethical considerations may preclude any steps to compromise the skin.

The ability to evaluate potential irritation using a variety of conditions under highly controlled circumstances has implications for products other than catamenial products. For example, with baby and adult diapers, the precise conditions encountered may be difficult to control in an in-use clinical study. Different use habits and patterns would influence the degree of moisture in the product. Confounding factors, such as pre-existing rashes or irritation, may exist and compromise the results. However, in the BTK, single variables can be controlled and evaluated within a short time, providing more precise results and increasing the ability to pinpoint any contributions of the product to the overall skin effects.

We have tested over 25 different materials in over 35 BTK studies, and the test method has proven reliable and versatile in testing a wide variety of materials, such as fabric, menstrual pads, topsheets, interlabial pads, pantiliners, tampons, and lotion coatings on products. Table 84.4 illustrates the utility of the BTK test method for testing various product types. It is possible to test products of different shapes and end uses, for example, externally worn catamenial pads, interlabial pads, and tampons, with no alterations to the basic protocol. For some materials, in-use clinical studies were available on the identical test products compared in the BTK test. These are given in Table 84.4a. In all cases, the BTK test gave results similar to the in-use study. For other materials, alternative data were available that enabled a prediction regarding the expected result in the BTK test (Table 84.4b). This alternative data included a knowledge of the nature of the materials being tested (as in the case of the fabrics burlap and satin), or evaluations and previous testing (as in the case of menstrual pads A and B, and topsheets AT and BT). In all cases, the BTK test gave the expected result. It is noteworthy that several of the BTK studies mentioned in Table 84.4 have been repeated several times, giving results consistent with those in the table.

A main use for in-use clinical testing is to establish the safety of a new product or product improvement by demonstrating that the test sample does not result in an increase in adverse skin effects compared to the control sample. For the products presented in Table 84.4a, clinical testing was conducted to demonstrate no change in the irritation potential for the product safety assessment, claims support, and for the purposes of regulatory filings. Given the nature of the in-use clinical test, it would not be ethical to conduct studies with materials that would be expected to produce frank irritation. Further, given the expense and logistical difficulties associated with the in-use clinical studies, the number of studies that can be conducted for purely investigative purposes is limited. However, we were able to select products that had been tested recently in the in-use clinical studies, and test these same products in the BTK test, thus generating side-by-side comparisons to complete the validation of the BTK test. In all cases, the BTK test duplicated the results observed in the in-use clinical study conducted on the same materials in terms of similarities and differences in irritation, based on the data presented.

The potential irritation that may be caused by catemenial pads is a combination of the inherent irritation potential of the chemical components of the products, and mechanical irritation that occurs as a result of wearing the products in close proximity to the skin for prolonged periods of time during normal movement and activity. The standard patch test method does not evaluate mechanical irritation and, therefore, provides an incomplete picture. Figure 84.2 illustrates the contribution of mechanical irritation to the overall irritation potential. As shown in this figure, when the same

products are tested on the same panelists under the same experimental conditions, the BTK test site almost invariably exhibits more irritation than the standard patch test site. This difference is due to the mechanical irritation caused by the friction of the test material against the skin.

The BTK test can also be used to compare two or more products, as shown in Table 84.5. Separate panels can be conducted concurrently, sharing a single common test material. The common test material acts as a control, allowing the statistical comparison of the test materials applied to the other test site. Such side-by-side product comparisons are not possible using in-use testing.

Investigative programs are difficult to conduct using in-use clinical testing. However, with the BTK test, we have conducted many investigative studies to aid product development efforts. One such program involved evaluating subjective sensations of irritant effects, such as feelings of itching, burning, sticking, etc., and correlating these so-called sensory effects with outward evidence of irritation. In some studies, we found an association between the sensory effects reported by panelists and the degree of irritation determined by scoring for erythema [11]. Additional details of this program are provided in a separate publication [18].

An additional example of an investigative program is quantitative lotion transfer assessments. In these experiments, we studied the transfer to the skin of lotion from products with topsheets composed of different materials. The BTK study design was capable of detecting differences in the amount of lotion that transferred from topsheets of different composition. This work will be the subject of a future publication.

The BTK test is the result of a program to develop a method for evaluating skin effects for products used in the urogenital region that eliminates the difficulties of the in-use clinical test without compromising the quality of the results. The specific advantages of the BTK test are summarized in Table 84.6. Both testing approaches provide reproducible and reliable results, and can discriminate between very similar products. However, the flexibility, ease of implementation, rapid turnaround time, and lower cost of BTK test make it a much more useful tool for safety testing, investigative programs, product development efforts, and claims support. This test system provides a potentially valuable alternative for evaluating the skin effects of any material where mechanical irritation may play a role in overall skin irritation and consumer satisfaction, with potential applicability for textiles, facial tissues, baby and adult diapers, and laundry products that may leave residues on fabrics.

TABLE 84.6
Comparison of Characteristics of the In-Use Clinical and Popliteal Fossa Test Systems

Characteristics	In-Use Clinical Test System	BTK System
Quality of results	Provides reproducible, reliable results.	Provides reproducible, reliable results.
	Capable of detecting subtle differences between very similar products.	Capable of detecting subtle differences between very similar products.
	Side-by-side product comparisons not possible. Each panelist can test only one product at a time.	Conducive to side-by-side product comparisons. Each panelist can test two products concurrently.
Ease of implementation	Cumbersome.	Simple.
	Staggered start necessary. Start date for each panelist must coincide with the menstrual cycle.	Start date is independent of the menstrual cycle.
	Panel size of at least 30 per product.	Panel size of 15–20 for two products.
	Panel must be composed of the specific target consumer type, that is, menstruating women for catamenial products, incontinent adults for adult diapers, etc.	Many products may be tested on healthy, male, and female adult volunteers.
	Grading is intrusive for panelists.	Grading is not intrusive (similar to the standard patch test).
Confounding factors	Panelists may have a broad range of pad wearing and hygiene habits that may impact results.	Wearing time and test conditions can be controlled.
	Results can be confounded by changes in microbial distribution during the menstrual cycle.	Results independent of changes in microbial distribution.
Turnaround time	Results available in a minimum of 4–5 weeks from study initiation due to staggered start. Start date for each panelist must coincide with the menstrual cycle.	Results are available 1–2 weeks after initiation.
Cost	Costly (≥$150,000–$200,000 per study).	Inexpensive (~$5000 per study).
Usefulness in investigative programs	Investigative programs are not practical due to high-cost and slow turnaround time.	Investigative programs are inexpensive and quick.
	Investigative studies, such as testing on compromised skin, cannot be easily incorporated into the protocol.	Investigative studies are easily incorporated into the protocol.

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85 Need for More Sensitive Tools as We Reach Limits of Our Ability to Detect Differences in Skin Effects from Mild Products

Miranda A. Farage

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85.1 INTRODUCTION

Testing for potential skin effects is a key part of the overall safety assessment and product claims support for many consumer products. The current approach employs exaggerating the concentration and duration of exposure using a variety of test protocols, including standard patch testing and extended product use tests, with expert clinical evaluation of erythema and dryness as a measure of irritation. For decades this approach has served the consumer products industry well in detecting differences in potential skin effects [1–4]. This approach has allowed for the precise assessment of product safety performance, and provided support for product innovations that have enabled the consumer to benefit from continuous product improvements.

Modern products in the tissue and paper products categories (including facial tissues, catamenial products, baby wipes, and baby and adult diapers) are inherently very mild to skin, and produce few adverse skin effects when tested even under very stringent conditions. However, recently, we have seen that current test methods may not be robust enough to evaluate perceived skin effects that may be important for consumer comfort during product use. This observation is consistent with feedback from consumers obtained via our toll-free number, indicating that consumers develop product preferences on the basis of perceived skin effects that are not always expected on the basis of results of objective skin effects testing in the laboratory (unpublished data). Further, sensory data collected from panelists participating in skin effects testing indicate that panelists can sometimes discriminate between products on the basis of how they feel during use, even when clinically evaluated skin effects (erythema and dryness) show no differences. This has been observed, for example, when testing catamenial products in the Behind-the-Knee (BTK) test, and when testing facial tissues in a recently developed nasal irritation model [5,6]. Others have reported similar experiences in exaggerated use tests. Simion et al. [7], testing personal cleansing products in an exaggerated arm washing model, found that panelists' descriptions of sensations, i.e., dryness, tightness, itching, etc., yielded information on potential skin effects beyond the visible clinical observations.

These observations lead to the conclusion that our traditional skin testing approaches are not always good predictors of consumer-perceived skin comfort. Many of our recent efforts have been directed toward increasing our capabilities to predict and quantify these consumer-perceived differences in comfort that are often undetected using the traditional skin-test methods and assessment tools. We have been exploring three approaches: (1) further exaggerating exposure conditions; (2) increasing the sensitivity of the manner in which we score for irritant effects, either visually or via instrumentation;

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and (3) quantitatively measuring additional endpoints, i.e., subjective sensory effects. Of course, any modifications or new methods must be cost-effective, and should not significantly increase the level of panelist discomfort. This is a commentary on the progress we have made in these areas.

85.2 FURTHER EXAGGERATING EXPOSURE CONDITIONS

During use testing of consumer products, exposure conditions can be exaggerated by increasing the concentration of the product during testing, or by increasing the duration of exposure. Neither of these approaches is practical for tissue and paper products, where products are intended for use undiluted, and where some products (catamenial products and baby and adult diapers) are intended to be worn in contact with the skin for prolonged periods of time. However, it is possible to exaggerate the conditions of use by increasing the mechanical irritation through friction at the test site, or by testing on skin that has been compromised by tape stripping or other means. Recently, we have conducted programs on three test methods that use one or both of these approaches.

85.2.1 Increasing Mechanical Irritation Through Friction

The BTK test was developed specifically for catamenial products [8,9]. Samples are applied daily to the back of the knee using an elastic athletic band for 6 h each day for 4–5 days. This sample application method results in increased mechanical friction compared to actual use conditions, with a resulting increase in the irritant reactions that develop. Irritation reactions are scored by visual assessment. The BTK test gives consistent and reliable results on different types of materials, from thin menstrual pad topsheets to tampons. Results from selected studies are summarized in Table 85.1. Comparisons to testing conducted at other body sites, such as the upper arm, axilla, and wrist, indicated that the BTK test produced the most reliable results, with the greatest ease of sample application, and the least discomfort to the volunteer panelists [8]. In addition, comparison of the BTK test results to in-use clinical tests conducted on the same materials indicates that the BTK test provides results that are consistent with the in-use clinical test in a shorter period of time and at a greatly reduced cost. In addition, the BTK is easier to conduct for both investigators and panelists.

A nasal irritation model was developed to study potential skin effects of facial tissues [6]. Like the BTK test, this model uses an application approach to increase the mechanical irritation over and above that which would be expected to occur during normal product use. In this system, panelists wipe the facial tissues (one on each side) from the nasal bridge to the upper lip for 10 wipes every 20 min for a total of 170 wipes (17 series) daily on Monday through Thursday. Four areas of the nose were visually scored for erythema and dryness: the nasal bridge, nostrils, nasolabial folds, and the area underneath the nostrils. In a comparison of a lotioned facial tissue to a nonlotioned facial tissue, differences were observed between the test products at isolated time points. Interestingly, in response to specific questions

TABLE 85.1
BTK Results on Diverse Product and Material Types

Exposure Regimen	Number of Panelists	Materials Tested	Mean Irritation Score ± SE	Comments
6 h/day for 4 days	12	Burlap fabric	1.5 ± 0.17**	Expected result based on material characteristics
		Satin fabric	0.6 ± 0.08	
6 h/day for 4 days	17	Standard pad A	1.7 ± 0.06**	Expected result based on previous testing and marketplace experience
		Standard pad B	1.1 ± 0.10	
16 h/day for 5 days	11	Topsheet AT	1.3 ± 0.25*	Expected result based on previous testing and marketplace experience
		Topsheet BT	0.59 ± 0.16	
16 h/day for 5 days	14	Minipad AU	1.5 ± 0.17*	Expected result since a lotion coating would be expected to reduce mechanical irritation
		Lotioned pad AUL	1.1 ± 0.19	
6 h/day for 5 days	15	Tampon R	0.90 ± 0.09[a]	Expected result based on the similarity of the two test products
		Tampon S	1.0 ± 0.10	
6 h/day for 5 days	22	Intralabial pad IL2	1.4 ± 0.09[a]	Expected result based on in-use clinical study results
		Pantiliner P	1.4 ± 0.11	

Note: Samples were applied daily to the back of the knee using an elastic athletic band for the specified number of hours each day for 4–5 days. This method of sample application results in an increase in mechanical friction compared to actual use conditions, with a resulting increase in the irritant reactions that develop. Irritation reactions at the test sites were scored by visual assessment on a scale of "0" (no irritation) to "4" (severe irritation) 30–60 min after removal of the test samples. The mean irritation score for each product was determined and evaluated using stratified Cochran–Mantel–Haenszel (CMH) tests.

[a] Not significant.

* Significant at $<5\%$.

** Significant at $<0.02\%$.

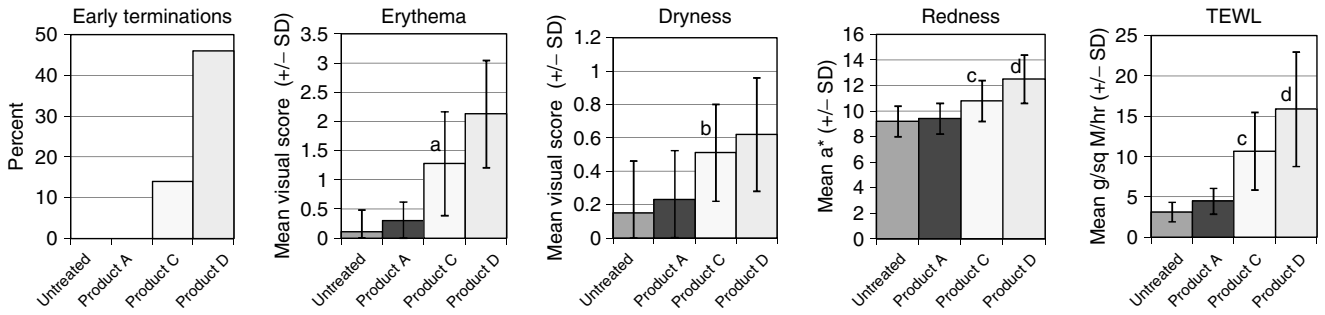


FIGURE 85.1 Comparison of three baby wipe products in the modified FCAT. Treatment consisted of 15–30 s wipes on the forearm using the baby wipe product four times each day for 5 days. Reactions were evaluated by visual scoring on a scale of "0" (no irritation) to "4" (severe irritation), and by instrumental scoring for TEWL and erythema. Early terminations indicate the percentage of panelists in which a visual score of ≥3 for erythema at the test site necessitated termination of the washing procedure prior to completion of the study. For the remaining endpoints, data for the final scores on the final day are given (mean ± SD). Thirty-one panelists completed the study. (a) Significantly different from product A ($p < .0001$) using Wilcoxon's matched pairs test. (b) Significantly different from product A ($p < .001$) using Wilcoxon's matched pairs test. (c) Significantly different from untreated control, and products A and D ($p < .01$) using Tukey's protected t-test. (d) Significantly different from untreated control, and products A and C ($p < .01$) using Tukey's protected t-test. (Adapted from Farage, M.A., *J. Cosmet. Sci*., 51, 153, 2000.)

on various sensory effects, panelists expressed product preferences statistically more significant than those detected by the objective measures for irritation, i.e., visibly scored erythema and dryness.

We are beginning experiments using an abrasion wheel device. This equipment uses a rotor, turning at speeds up to 100 rpm, to lightly rub the skin. When a tissue or paper product is affixed to the surface of the wheel, the product can be evaluated for mechanical irritation effects. Reactions are scored visually, and via a laser Doppler scanner. Results will be the subject of a future publication.

The modified forearm controlled application technique (modified FCAT) was developed to evaluate the relative mildness of several types of products, such as baby wipes, feminine hygiene products, and facial tissues [10]. Treatment consisted of 15–30 seconds wipes on the forearm using the baby wipe product four times each day for 3–5 days. Reactions were evaluated by visual scoring, and by instrumental scoring for transepidermal water loss (TEWL, using an Evaporimeter EP1®, ServoMed, Sweden) and erythema (using a Chromameter CR-200®, Minolta, Japan). The method demonstrated consistent and clear product differences by all scoring methods. Results of a typical study are shown in Figure 85.1.

85.2.2 Testing on Skin That has been Compromised by Tape Stripping or Other Means

Surprisingly, tape stripping skin test sites prior to exposure has not resulted in an increase in the ability of our test models to demonstrate differences in the potential irritant effects of our products. Tape stripping was used in the development stages of the BTK test described above, but did not increase the ability of the test to detect product differences compared to skin that had not had this pretreatment. In the nasal irritation test for facial tissues, tape stripping was done on one of the four areas prior to treatment—the nasolabial fold. If tape stripping increased the sensitivity of the test, one would expect that product differences would show up more frequently at this site, compared to the other three sites that were not pretreated in this manner. However, few product differences were detected, and those that were evident occurred randomly at all four of the anatomical sites that were graded for erythema and dryness.

There may be several explanations why tape stripping has not increased the sensitivity of our tests. One may be that our tape stripping protocol was not vigorous enough. In our models, we used up to 20 repeated applications of tape. Bashir et al. [11], using TEWL to study barrier disruption in skin stripped multiple times, found that TEWL did not increase significantly after 10 or 20 tape applications. After 30 applications, TEWL had increased significantly on the dorsal surface of the forearm, but not the ventral surface. A significant increase on the ventral surface was observed only after 40 sequential applications.

Another possible explanation why tape stripping may not have had an impact on the sensitivity of our tests may be the specific tape being used. Different tape adhesives may have different affinities for materials within the stratum corneum, leading to potential differences in the impact of tape stripping. Schwindt et al. [12] proposed that water diffusion through the stratum corneum is determined by the intercellular lipids, and that the corneocytes are minimally permeable. Thus, use of a tape with an adhesive that has a high affinity for lipids may result in an increased permeability for hydrophilic materials. Conversely, use of a tape with adhesive that has a low affinity for the intracellular lipids may lead to removal of corneocytes, and an increased permeability to hydrophobic materials.

There is also evidence that pretreatment to compromise the barrier function of the skin may lead to a reaction that is not typical of the classical irritant response. Gebhard

et al. [13] used a number of pretreatment protocols prior to application of sodium lauryl sulfate (SLS). These included (1) none, (2) pricking with a lancet, (3) pricking with a test stamp, having four single pricks, (4) scarification with a lancet, and (5) incision scratching. Interestingly, the site with no pretreatment produced the highest clinical scores for irritation reactions. Further, there was a qualitative difference between the reactions produced either with no pretreatment or with the first two pretreatment protocols (i.e., treatments 1–3, above) compared to the latter two pretreatment protocols (treatments 4 and 5). The proposed explanation was that the more aggressive pretreatments produced reactions in the deeper layers of the epidermis that did not reflect the reaction that occurs with a topically applied irritant. Granted, tape stripping compromises the stratum corneum in a way that is different from puncturing or incising the epidermis. However, such results would suggest caution when using skin-compromising pretreatment techniques in the development of test models for consumer products intended for use on intact, healthy stratum corneum.

85.3 INCREASING SCORING SENSITIVITY (EITHER VISUALLY OR VIA INSTRUMENTATION)

Visual scoring represents the cornerstone of skin irritation testing, and trained skin graders can accurately and reproducibly score test sites for erythema and dryness. Griffiths et al. [14] conducted an intralaboratory comparison of results from patch testing using standard irritants. By necessity, different graders were used. There was excellent correlation between results obtained at the different laboratories. Several authors have demonstrated that trained graders can reliably detect evidence of irritation with degrees of sensitivity equal to or higher than that of instrumental measures [15–19]. Results from our own laboratory indicate that visual scoring yields results that are as reliable as measures of TEWL or of erythema via chromameter.

It is possible that the design of many current test models is not optimum to detect early, subclinical changes in the skin. Current test methods were developed to result in a grader being able to see a reaction. However, physiological changes that occur early in the process of irritation, such as changes in blood flow, moisture content, pH, etc., would be expected to occur before any reaction is visible. In other words, by the time the reaction is visible, it may be too late to measure the early changes in skin physiology. These early changes may be key to our ability to distinguish subtle skin effects and therefore support future product development efforts However, in order to see these early changes, investigators must either design a different type of test or use nonirritating materials in existing tests.

Reports from some authors support this notion. In the examples given above, visual scoring of irritant reactions was equally or more reliable than some instrumental methods [15–19]. However, some investigators have described measurable changes in skin characteristics after treatment with materials that produce very little or no visible reaction. In patch testing using moisturizer formulations, Agner et al. [20] scored reactions for redness (visual), TEWL, blood flow (via laser Doppler), and hydration (via electrical capacitance). Test sites were scored prior to patching, after one 24-h patch (day 1), and after two 24-h patches (day 2). Reactions at test sites were compared to a positive control (SLS) and negative control (empty patch chamber). They showed detectable differences in skin hydration and blood flow, but nothing detectable by TEWL or visual scoring. Tupker et al. [21] evaluated the irritant properties of six antiseptics using twice daily open exposures. Reactions were evaluated by visual scoring, subjective irritancy scoring, skin hydration, TEWL, and laser Doppler. All evaluation methods identified the same two antiseptics as the most irritating. However, the rank order for the other four materials varied, depending on the scoring method. In this example, different instruments were likely measuring different physiological parameters that may not have been reflected in the visible signs of irritation.

Several methods are available to quantitate various changes in skin physiology that may occur early in the irritation process or in the case of subclinical reactions. Many of these have been reviewed recently by Berardesca et al. [22]. In our laboratories, we have been investigating several for possible use in our skin effects testing.

An area of investigation we have undertaken recently is instrumental enhancement of visual scoring through the use of polarized light. In this method, the skin is examined using parallel-polarized light, which illuminates surface detail, and cross-polarized light, which allows examination of subsurface details, such as the vasculature [23]. Authors have described the use of polarized light as an aid in visualizing various skin conditions, including acne vulgaris, rosacea, photo-aging, lentigo simplex, and basal cell carcinoma [24–26]. Recently, we have conducted preliminary experiments using a system that allows the visualization of the skin using both parallel- and cross-polarized light. Results will be reported in a future publication.

Another area we have explored is the measurement of cytokines. Since cytokines are important mediators of inflammation, the ability to quantitate changes in cytokine levels could provide important markers for the inflammatory process. We used a tape absorption method to isolate and quantitate the presence of cytokines in normal skin, as well as in skin that was compromised by diaper or heat rash, sun exposure, or treatment with a chemical irritant (SLS) [27]. Cytokines were collected via a 1 min application of Sebutape® (CuDerm Corporation, Dallas, TX), extracted from the tape using saline, and evaluated using immunoassay techniques. Extracts were analyzed for total protein and for the cytokines interleukin (IL)-1α, IL-1RA, and IL-8. In skin that was affected by diaper or heat rash, we saw significant increases in IL-1α levels (determined as a percentage of total protein) compared to uninvolved skin. In sun-exposed skin, we found significant increases in the IL-1RA/IL-1α levels (three- to six fold) compared to skin that typically receives minimal sun exposure. In skin treated with SLS, under conditions that

TABLE 85.2
Analysis of Perceived Sensory Effects versus Irritation Scores in the BTK

	Study 1			Study 2		
	Mean Irritation Score			Mean Irritation Score		
Sensory Effect	Complaint Reported by Panelist	Complaint Not Reported	Regression Results (*p* Value)	Complaint Reported by Panelist	Complaint Not Reported	Regression Results (*p* Value)
Burning	1.70	1.50	.069	1.82**	1.50	<.001
Sticking	2.25**	1.52	.059	1.75**	1.54	.001
Pain	1.88	1.52	.155	1.78**	1.57	.019
Itching	1.52	1.53	.938	1.70*	1.58	.148
Rubbing	1.52	1.53	.949	1.67*	1.58	.187
Chafing	1.33	1.54	.308	1.65	1.58	.356
Other	1.46	1.53	.613	1.79**	1.58	.040
Edema	None reported	NA	NA	1.17**	1.60	.008

Note: The studies were conducted as described in Table 85.1. The mean irritation scores were determined for all test sites where the sensory effect was reported by the panelist, and for all sites where the effect was not reported. Significant differences between the means are indicated (*$p \leq .10$, **$p \leq .05$). Irritation scores were then evaluated versus reported sensory effects using logistic regression, i.e., the presence/absence of a particular skin complaint was regressed against the irritation scores. Burning and sticking showed a relationship to the irritation score in both experiments. Pain and other (nonspecified) complaints showed a relationship to the irritation score in Study 2. The reported complaint of edema showed an inverse relationship to the irritation score.

caused a minimal visible irritation reaction, we saw significant increases in IL-1α levels, directional increases in IL-8 levels, and significant decreases in the IL-1RA/IL-1α ratio.

Instrumental measures of barrier function (TEWL) and redness (via chromameter) have demonstrated little advantage over visual scoring in our test systems. As mentioned above, both methods were used in the modified FCAT. Results were similar to those obtained with visual scoring. Other investigators have had similar experiences, as mentioned in the previous sections of this article. In addition to use in the modified FCAT, we have tried TEWL on a number of body sites. The necessity for equilibration in a temperature- and humidity-controlled room prior to the measurement increased the complexity of our scoring process, while providing no real advantage.

85.4 QUANTITATIVELY MEASURING ADDITIONAL ENDPOINTS, I.E., SENSORY EFFECTS

Sensory effects have been a critical area of investigation for some time for cosmetic preparations, where unpleasant sensations during product use can be a determining factor in consumer dissatisfaction. Recently, we have developed an increasing interest in using sensory effects in evaluating other types of consumer products [5,6]. As mentioned in some of the examples above, several investigators have described test results where panelists are able to differentiate between different products based solely on sensory effects.

Measuring sensory effects in tests for skin irritation is developing increasing importance, especially for subclinical reactions. Green [28] reviewed some important considerations in designing tests to quantitate sensory effects, including the use of the Labeled Magnitude Scale (LMS). Robinson and Perkins [29] subsequently used this scale to evaluate subject responses in chemosensory skin irritation studies using lactic acid and capsaicin.

Adding quantitative measures around sensory effects to our current test protocols may provide a potentially valuable tool for us to increase our abilities to detect product differences. Although our current test methods have not been optimized for this endpoint, we have begun to evaluate sensory effects in a number of recent studies.

In the BTK test mentioned above, panelists were asked questions on specific sensations they experienced at the test sites: sensations such as burning, itching, sticking, etc. [8]. We determined that the product that was more irritating according to objective scoring also produced a higher number of certain skin complaints from the panelists. In addition, the mean irritation scores for the skin test sites where panelists complained of certain sensory effects was significantly higher than for those sites where the panelists did not have the specific complaint. This result is illustrated in Table 85.2.

We correlated the panelists' sensory experiences in two BTK tests with data generated as part of the routine product development process, i.e., the Descriptive Analysis Panel (DAP) [5]. This panel is a means of evaluating positive and negative physical characteristics of materials being considered for use in certain products intended to come into contact with the skin, such as diapers and catamenial products. In the DAP, specifically trained individuals grade products on characteristics such as scratchiness, cottony feel, plastic feel, smoothness, etc. In one product comparison, where visual scores and sensory scores produced a clear differentiation between the two products, we found a correlation with the DAP results for those two products. In a second

comparison of two products, we found mixed results in visual scores, sensory scores, and DAP results.

Like the BTK studies, the nasal study on facial tissues mentioned above was not designed to optimize the ability to detect differences in sensory effects [6]. Nevertheless, the panelists expressed a clear preference for the lotioned product over the nonlotioned product on the basis of a number of sensory effects endpoints, even though only small differences were observed in the visual measures of irritation.

85.5 CONCLUSION

Current test methods have been used successfully for decades to detect differences in potential skin effects in the course of product development activities and claims support. Our challenge for the future is to use all potential tools in our arsenal for developing more sensitive test methods capable of detecting skin benefits and very subtle improvements in the potential for adverse effects. These tools include seeking ways to further exaggerate exposures, enhancing the ability to clinically score for the endpoint of irritations, either visually or via instrumentation, and finding new scoring endpoints, such as subjective, sensory effects.

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86 Drug Patch Testing in Systemic Cutaneous Drug Allergy

Annick Barbaud

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86.1 INTRODUCTION

Cutaneous adverse drug reactions (CADRs) are a frequent problem in clinical medicine. Since patients are often on multiple drug regimes, it is often difficult to pinpoint the relevant drug from history alone. Beside clinical and chronological parameters, there is no standard complementary test to help in defining the cause of the CADR.

Patch testing with the suspected compound has been reported to be helpful in determining the cause of a CADR (Barbaud et al., 1998b, 2000, 2001a; Osawa et al., 1990; Bruynzeel and Van Ketel, 1987, 1989; Barbaud, 2002, 2003; Bruynzeel et al., 1985; Romano et al., 1993) and in studying the pathophysiological mechanisms involved in these reactions. The results of drug patch tests depend on the drug tested and the clinical features of the initial CADR (Barbaud et al., 1998b; Osawa et al., 1990; Bruynzeel and Van Ketel, 1987). There are, at present, only a few extensive studies that determine the sensitivity and specificity of these drug skin tests as a complementary tool for drug imputability in CADR.

The main advantages of drug patch tests are that they can be done with any commercialized form of drugs while intradermal tests (IDT) need to be performed with an injectable form or with a pure and sterile preparation of the drug. Patch tests can be done with no hospital surveillance because they only rarely induce adverse reactions, which are mild.

86.2 DRUG PATCH TESTS CAN REPRODUCE THE MECHANISMS INVOLVED IN SOME CADR (BARBAUD, 2002)

On biopsy samples of maculopapular rash (MPR) and on biopsy taken from positive patch tests with amoxicillin obtained in the same patients, it was observed that drug patch tests can reproduce the immunological mechanisms involved in CADR due to betalactam antibiotics. Similar expressions of adhesion molecules such as ICAM-1 (CD54) on keratinocytes or ELAM-1 (CD62E) on endothelial cells were observed on biopsy sample taken from an MPR or a positive patch test (Barbaud et al., 1997).

Expression of CD62L was found in 3/4 cases of acute erythrodermia and 1/3 MPR due to pristinamycin. This finding could be confirmed in one patient with erythrodermia, as the biopsy from a positive patch test with pristinamycin also showed higher expression of CD62L (Barbaud et al., 1998a).

Immunophenotyping of patch tests of patients with acute generalized exanthematous pustulosis (AGEP) revealed similar results of a skin cell infiltrate in patch and acute skin lesions: T cell expressed mainly CD4 and produced interleukin 8 (IL-8), which is a chemokine attracting neutrophilic leukocytes and can cause neutrophilia, as it is observed in AGEP (Britschgi et al., 2001).

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86.3 HOW TO PERFORM DRUG PATCH TESTS?

It is advised to perform drug patch tests during the 6 months following the CADR, as we do not know whether positive results will persist, and whether some drug reactivity will last longer (Barbaud et al., 2001a). Guidelines for performing skin tests (patch tests, prick tests, and IDT) with drugs in the investigation of CADR have been published based on the experience of dermatologists belonging to the working group of the European Society of Contact Dermatitis for the study of skin testing in investigating CADR and from a review of literature (Barbaud et al., 2001a).

Due to the possibility that a low concentration might yield false-negative results, drug patch tests have to be performed with high concentrations of the commercialized form of the drug. Using a pulverized tablet, 30% is the highest concentration possible to get a homogeneous dilution in petrolatum, in water, or in alcohol (Barbaud et al., 2001a).

Testing with acyclovir, carbamazepine, or pseudoephedrin has been reported to reinduce the CADR symptoms during patch testing. Therefore, it is recommended that patch tests are performed, first diluted at 1% and when negative, up to 10% (either with the commercialized form of the drug or the pure substance). Similarly, to avoid false-positive reactions with Celebrex®, the content of capsules should be tested at 5% or at 10% in petrolatum and not with higher concentrations.

Since the threshold of sensitivity for many pure substances is not yet determined, it is advised to use a 10% concentration in petrolatum and if necessary in other vehicles, although for some drugs smaller concentrations may be sufficient.

If the commercialized form of the drug is tested, pills must have their coating removed. The substance has to be smashed to a very fine powder. This powder can then be tested as it is, but has to be incorporated at 30% in white petrolatum and also be diluted at 30% in water.

The powder contained in capsules is tested at 30% in petrolatum and at 30% in water. The gel jacket portion of the capsules is moistened and tested as it is. Liquid preparations are tested both as it is and diluted at 30% in water. With commercialized forms of the drugs, each preparation is done for only one patient and kept no more than 24 h. The name of the chemical form of the molecule (salt, molecule base) has to be noted carefully. To avoid any relapse of a severe CADR in patients who have developed drug rash with eosinophilia and systemic symptoms (DRESS), Stevens Johnson syndrome, Lyell's syndrome, generalized urticaria, angioedema, and especially in case of anaphylactic shock, it is recommended that patch tests are performed with more diluted substances, for example, first diluted at 0.1% and when negative, at higher concentrations, 1%–10%.

Whenever possible, preservatives, coloring agents, and excipients should also be tested, undiluted or diluted at 10% in petrolatum, or in the vehicles and concentrations usually proposed for testing allergic contact dermatitis. Delayed CADR due to excipients are very rare (Barbaud, 1995). Positive patch tests with excipients have been observed in one case with sorbic acid (Giordano-Labadie et al., 1996) and in an MPR due to xanthan gum contained in syrup (Barbaud, unpublished data).

In investigating a photosensitivity reaction induced by a drug, both drug patch tests and drug photopatch tests with the responsible drug have to be performed. The irradiation for drug photopatch tests is performed on day 1, or for practical reasons can be performed on day 2 with a 5 J/cm^2 UVA irradiation (Barbaud et al., 2001a).

To prepare drug patch tests it is proposed to use different vehicles, at least petrolatum and water, to avoid false-negative results due to a low penetration of the drug into the epidermis. False-negative results can occur when antibiotics such as betalactam antibiotics or pristinamycin (Barbaud et al., 1998b) are diluted in water, whereas patch tests with the same drug diluted in petrolatum yield positive results (Barbaud et al., 2001a). Steroid hormones have to be tested by diluting in alcohol, as false-negative results have been observed in testing estrogens diluted in water or petrolatum (Goncalo et al., 1999). We also observed an MPR with positive patch tests with corticosteroids diluted in water and alcohol but negative when diluted in petrolatum (Barbaud, unpublished data). Positive patch tests were obtained with ganciclovir diluted at 20% in water but negative with the drug diluted at 20% in petrolatum (Lammintausta et al., 2001). There are no extensive studies concerning the value of other vehicles used in drug patch tests.

Patch tests are performed on the upper back but it could also be of value to test on the most affected site of the initial CADR. In fixed drug reaction (FDE), patch tests (Alanko et al., 1987) or repeated application tests (Alanko, 1994) with the suspected drug are positive only when performed on the residual pigmented skin site of the CADR but not when applied on the nonpreviously affected skin of the back.

Testing in the affected area could also be of value in other forms of CADR such as toxic epidermal necrolysis. Klein et al. (1995) obtained positive patch tests when co-trimoxazole was tested on the cutaneous sites previously affected by necrolysis while drug patch tests performed on other less affected skin sites remained negative. It could also be of value to test on the most highly affected skin sites in MPRs. In a woman who twice developed a MPR due to tetrazepam with bullous lesions on the elbows, patch tests were negative when performed with this drug on the back but had positive results when tested on the elbows, the most highly affected skin sites. No cross-reactions were observed with diazepam that has a chemical structure close to that of tetrazepam (Barbaud et al., 2001c).

The results of patch testing are reported according to the International Contact Dermatitis Research Group (ICDRG) criteria for patch test reading (Wilkinson et al., 1970).

As drug patch tests can induce immediate positive reactions, especially with betalactam antibiotics, these tests have to be read at 20 min, especially in patients who have developed urticaria or anaphylactic shock. Immediate reactions

on patch tests have been reported with betalactam antibiotics, neomycin, gentamycin, bacitracine, and recently with diclofenac (Jonker and Bruynzeel, 2003). Immediate positive results can be associated with generalized anaphylactic reactions.

Because most of the CADR are related to a delayed cellular hypersensitivity, it is absolutely necessary to do delayed readings at 48 h, at 96 h and, if negative, on day 7. Patch tests can be negative when read on day 2 but positive on day 4 (Barbaud et al., 1998b).

86.4 THE CLINICAL SIGNIFICANCE OF DRUG PATCH TESTS

In patients with a high imputability that a certain drug causes the CADR, drug (photopatch) patch tests had positive results in 43% of 72 patients (Barbaud et al., 1998b), 50% of 108 patients (Barbaud et al., 2000), or 31.7% of 197 patients (Osawa et al., 1990).

When they are negative, it is advisable to perform prick tests, and if these are negative, IDT with immediate readings in urticaria but with delayed readings in other CADR can be conducted (Barbaud et al., 1998b, 2001a; Romano et al., 2002). Among 60 patients with CADR and negative patch tests with the suspected drug, 35 (58%) had positive results on IDT (Barbaud et al., 1998b). Among 94 patients with a suspected delayed sensitization to betalactam antibiotics, 36% had both positive patch tests and IDT but eight had positive IDT with negative patch tests (Romano et al., 2002).

In a CADR due to vancomycin, prick tests as well as IDT done with glycopeptide antibiotics remained negative even on delayed readings while drug patch tests were positive with specific results (20 negative controls) (Bernedo et al., 2001). This case emphasizes that delayed reactions to IDT are not sufficient to investigate a CADR due to delayed hypersensitivity to drugs, but that drug patch tests have also to be performed as they can be positive while IDT remains negative.

The usefulness of drug patch tests depends on the clinical features of the CADR. In 165 patients suffering from a CADR, with a high imputability of one drug, patch tests were positive in 33/61 (54%) MPR but in only 2/33 (6%) of urticarias, and the difference was statistically significant (Chi square test) (Barbaud et al., 2000). Patch tests were positive in 7/14 AGEP (50%) but only in 2/22 patients with Stevens–Johnson or Lyell syndrome (Wolkenstein et al., 1996).

Patch tests are of value in determining the responsible drug in generalized eczema, systemic contact dermatitis, Baboon syndrome, MPR (Bruynzeel and Van Ketel, 1987; Barbaud et al., 1997, 2000, 2001a; Barbaud, 2002; Romano et al., 1995), AGEP (Wolkenstein et al., 1996), and FDE (Alanko et al., 1987). They also seem to be valuable in DRESS. Photopatch tests may be useful in studying drug photosensitivity.

On the other hand, they are of less value in investigating urticaria (Barbaud et al., 1998b), Stevens–Johnson or Lyell syndromes (Wolkenstein et al., 1996), pruritus or vasculitis (Barbaud et al., 2000). The usefulness of drug patch tests also depends on the tested drug.

The most frequent reports of positive drug patch tests are related to the following drugs: betalactam antibiotics (Romano et al., 2002), especially amoxicillin (Barbaud et al., 1997, 1998b, 2000; Bruynzeel and Van Ketel, 1987, 1989; Bruynzeel et al., 1985; Romano et al., 1993, 1995; Vega et al., 1991; Felix and Comaish, 1974), cotrimoxazole (Klein et al., 1995), corticosteroids (Barbaud et al., 1998b; Dooms-Goossens, 1995; Bircher et al., 1996; Kilpio and Hannuksela, 2003), heparin derivatives (Barbaud et al., 1998b; Bircher et al., 1990; Moreau et al., 1996; Figarella et al., 2001), pristinamycin (Barbaud et al., 1998b, 2004; Michel et al., 1996), carbamazepine (Barbaud et al., 1998b, 2000; Vaillant et al., 1989; Silva et al., 1982; Camarasa, 1985; Romaguera et al., 1989; Jones et al., 1994; Rodriguez-Mosquera et al., 1991; Alanko, 1993; Corazza et al., 1995; Puig et al., 1996), diltiazem (Barbaud et al., 1993, 1998a; Romano et al., 1992; Cholez et al., 2003), hydroxyzine (Barbaud et al., 1998b; Michel et al., 1997), pseudoephedrine (Barbaud et al., 1998b; Tomb et al., 1991; Sanchez et al., 2000), or tetrazepam (Barbaud et al., 1998b, 2001c; Camarasa and Serra-Baldrich, 1990; Collet et al., 1992; Tomb et al., 1993; Calkin and Maibach, 1993; Pirker et al., 2002).

Miscellaneous drugs have been reported as yielding CADR with positive drug patch tests (Barbaud et al., 1998b; Barbaud, 2003): acyclovir and valaciclovir (Lammintausta et al., 2001; Vernassiere et al., 2003), allylisopropylacetylurea in an FDE (Sakakibara et al., 2001), captopril (Barbaud et al., 1998b), cefcapene pivoxil (Kawada et al., 2001), celecoxib (Britschgi et al., 2001; Verbeiren et al., 2002), clobazam (Machet et al., 1992), codeine diluted at 1 and 5% in petrolatum (two negative controls) (Estrada et al., 2001), co-trimoxazole (Klein et al., 1995), cyamemazine (Barbaud et al., 1998b), diazepam (Felix and Comaish, 1974), diclofenac (Jonker and Bruynzeel, 2003; Romano et al., 1994), enoxoparin (Barbaud et al., 1998b), estrogens (Gonçalo et al., 1999), fluoroquinolones (Barbaud et al., 1998b; Rodriguez-Morales et al., 2001), fusafungine (Barbaud et al., 1998b), isoniazide at 50% in petrolatum (10 negative controls) (Rebello et al., 2001), Lamisil® (Barbaud et al., 1998b), meprobamate (Barbaud et al., 2001a), metamizole at 1 and 10% in petrolatum (Gonzalo-Garijo et al., 2001; Quinones Estevez and Fernandez Schmitz, 2001), mexiletine hydrochloride diluted at 10 and 20% in petrolatum (Sasaki et al., 2001), nystatin at 10% in petrolatum (10 negative controls) (Barranco et al., 2001), paracetamol (acetaminophen) (Mashiah and Brenner, 2003), and vancomycin at 0.005% in water (Bernedo et al., 2001).

Patch tests could be of value in investigating delayed CADR occurring after an injection of nonionic radio contrast medium (RCM) (Brockow et al., 1999). In a study by Akiyama et al. (1998), 46/58 patients with delayed CADR had positive patch tests with RCM. We have also observed such positive patch tests with different RCM even though IDT with delayed readings are more frequently positive in patients suffering from delayed reactions after receiving RCM (Vernassiere et al., 2004). We obtained two positive

patch tests in 15 patients with delayed reactions to RCM, but 8/15 had delayed positive reactions on IDT.

86.5 PATCH TESTS TO STUDY CROSS-REACTIVITY BETWEEN DRUGS

Patch tests can help to study the ability of drugs to elicit symptoms due to cross-reactivity, for example, between betalactam antibiotics (Romano et al., 2002). In MPR due to diltiazem, patch tests with other calcium channel blockers are rarely positive, and no cross-reactions between dihydropyridine CCB and "nondihydropyridine" CCB are found (verapamil, diltiazem) (Cholez et al., 2003).

No or rare cross-reactivity between tetrazepam and diazepam was detected in two recent studies (Pirker et al., 2002; Weber-Muller et al., 2004). Cross-reactions between acyclovir, valacyclovir, and famciclovir are frequent in patients suffering from CADR due to acyclovir (Lammintausta et al., 2001; Vernassiere et al., 2003). It could be due to common chemical structure, as the 2-aminopurine nucleus is also found in ganciclovir but not in foscarnet or cidofovir (Vernassiere et al., 2003). Cross-reactions between synergistins are very frequent as demonstrated in 29 cases of CADR due to pristinamycin (Barbaud et al., 2004). In piroxicam reactions, the profile of cross-sensitization may differ in distinct clinical features of the CADR. In photosensitization, photopatch tests with piroxicam were positive in 27/31. But there was no cross-reaction with tenoxicam and lenoxicam and only 1/31 had positive photopatch tests with meloxicam. However, in eight patients with FDE, cross-reactions between oxicams were very frequent (Barbaud et al., 1998b, 2000; Romano et al., 2002; Goncalo and Figueiredo, 2002).

86.6 PREDICTIVE NEGATIVE VALUE OF DRUG PATCH TESTS

The predictive value of a negative patch test is unknown. When possible, if the imputability of a drug is probable in the onset of a CADR and if drug patch tests with this drug are negative it is advised to perform drug prick tests, followed by IDT with immediate and delayed readings. There are a lot of published cases of CADR with negative drug patch tests but positive delayed IDT (Barbaud et al., 1998b, 2000). It was also recently reported with rifampicin (Strauss et al., 2001).

Recently, we could demonstrate in delayed reactions occurring with RCM that the negative predictive value is low as with negative patch tests and IDT, 5/12 patients had a relapse during the readministration of an RCM that had negative skin tests (Vernassiere et al., 2004).

Therefore, it is advisable to perform first drug patch tests, then, if negative, the other drug skin tests, especially in CADR related to a delayed cellular hypersensitivity. We emphasize that there is no value for the patient in performing patch tests when positive delayed reactions have been previously obtained in testing the same drug with IDT. In such cases, drug patch tests are also very frequently positive.

86.7 SAFETY OF DRUG PATCH TESTS

Drug patch tests can reinduce the CADR. It has been reported with acyclovir (Barbaud et al., 1998b), amoxicillin and other betalactam antibiotics (with immediate reactions in case of anaphylaxis), carbamazepine (Vaillant et al., 1989), clobazepam (Machet et al., 1992), diclofenac at 1% petrolatum in an anaphylactic shock (Jonker and Bruynzeel, 2003), hydroxyzine (Barbaud et al. 1998b), metamizole (Gonzalo-Garijo et al., 2001), maybe due to paracetamol (acetaminophen) in an AGEP (Mashiah and Brenner, 2003), pristinamycin (Barbaud et al., 1998b), pseudoephedrin (Barbaud et al., 1998b; Tomb et al., 1991; Sanchez et al., 2000), and triamcinolone (Barbaud et al., 1998b).

86.8 RELEVANCE AND SPECIFICITY OF DRUG PATCH TESTS

One crucial point is the interpretation of the results of skin tests with drugs. As negative control, the vehicle used to dilute the material for patch tests should be tested: false-positive drug patch tests are rare but can be observed due to a sensitization not only to ethyl alcohol but also to petrolatum (Ulrich et al., 2004). False-positive results are rather frequent in performing IDT (Barbaud et al., 2001b), but false-positive or nonrelevant results can also be obtained in drug patch testing, namely, with the commercialized form of drugs containing sodium lauryl sulfate in their formulation. With colchicine diluted at 10% in petrolatum, 80% of 29 negative controls developed false-positive results. When diluted at 30% in petrolatum, Cytotec® containing misoprostol yielded false-positive results in nine of the 10 negative controls at the day 2 reading; however, none of the controls had false-positive patch tests at the day 4 reading or when Cytotec was diluted at 1% in petrolatum (Barbaud et al., 2001b). False-positive results have been obtained when testing with captopril, chloroquine, and omeprazole (Barbaud et al., 2001b).

Recently, Kleinhans et al. (2002) reported on the irritant reactions observed while patch testing Celebrex (celecoxib) with concentrations higher than 10% in petrolatum. Eight out of 10 patients with a suspected CADR due to Celebrex (all with negative oral provocation test) and nine patients without history of adverse reaction to Celebrex had false-positive results on the 48-h readings of their patch tests. If a 5 or 10% solution in petrolatum was used, patch tests with Celebrex seem to be specific.

This emphasizes the necessity to compare skin test results of patients with those obtained in negative controls (Barbaud et al., 1998b, 2001b), namely, patients who have taken the drug within the last 6 months without adverse effects and individuals who have never had contact with the culprit drug. However, such investigations need the signed informed consent.

A drug patch test may be positive due to a contact dermatitis to a drug or excipient without any relevance to the CADR (Barbaud et al., 2001b). In two patients, drug patch tests were positive with a commercialized form of a drug

and to a component of this product (iodine in one case, avocado oil in the second case), but the oral readministration of the drug was well tolerated. Drug patch tests were positive because the patients had previously developed a contact eczema due to an antiseptic containing iodine or to avocado oil contained in a wound-healing ointment (Barbaud et al., 2001b). Similarly, we observed positive results of patch tests with commercialized forms of drugs, where preservatives or stabilizers were causative, but not the drug itself, for example, sodium sulfite in certain formulation (Barbaud, 2003; Barbaud et al., 2001b) or benzyl alcohol in patients with heparin intolerance: these patients had previously developed contact allergy either to sulfites or to benzyl alcohol but had a good tolerance to systemically administered drugs containing these excipients.

86.9 CONCLUSION

Drug patch tests can be helpful in determining the cause of a CADR. They induce only rarely adverse reactions and they can be done with any commercialized form of a drug. A negative patch test does not exclude its role in causing a CADR. Their sensitivity seems to be somewhat lower than the one with IDT; on the other hand, patch tests can be positive in patients with negative IDT. Moreover, IDT cannot be done with all the commercialized forms of the drugs. False-positive results can occur and should be considered by testing new products. The specificity and their negative predictive value of patch tests have not yet been determined.

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87 Hormesis and Dermatology

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87.1 INTRODUCTION

Biphasic dose response, namely a low-dose stimulatory and a high-dose inhibitory response, also called hormesis in the field of toxicology, has been noted in a wide range of biological model systems from immunology to cancer biology [1–4]. Calabrese [1–4] has been the mainstay in bringing the attention of the scientific community to this interesting and a not uncommon phenomenon. As noted by Calabrese, the quantitative features of the hormetic-like biphasic dose response were remarkably similar with respect to the amplitude of the stimulatory response, the width of the stimulation, and the relationship of the maximum stimulatory response to the zero equivalent point (ZEP, i.e., threshold). Typically, the low-dose hormetic biphasic dose response stimulation is modest, with maximum stimulation between 30 and 60% greater than controls, and has a rather similar appearance in different cell types with various chemicals [4]. Most stimulatory ranges were less than 100-fold (averages 10- to 20-fold) measuring back from the ZEP. The low-dose stimulatory response often occurs following an initial disruption in homeostasis and appears to represent a modest overcompensation response. It is believed that the modest stimulatory responsiveness is due to a compensatory process that "slightly" overshoots its goal of the original physiological set-point, ensuring that the system returns to homeostasis without unnecessary and excessive overcompensation [5]. Therefore, it is important to follow the dose–response relationships over time to better define its quantitative features. While initial interest focused on the hormetic effects of pollutants and toxic substances on biological systems [6], the interest expanded to include pharmacological agents, phytocompounds, as well as endogenous agonists [4]. The hormetic-like biphasic dose–response relationships appear to be highly generalizable; that is, such responses do not appear to be restricted by biological model, endpoint, or chemical/physical stressors [4].

Many investigations attempted to assess mechanisms that could account for the hormetic-like biphasic dose–response relationship. In general, there is no single mechanism that accounts for the plethora of hormetic relationships. Nonetheless, a common molecular tactic by which biphasic dose–response relationships are displayed involves the presence of two receptor subtypes affecting cell regulation, one with high and the other with low affinity for the agonist but with notably more capacity (i.e., more receptors) [4]. Such an arrangement may lead to the biphasic dose response, with the high-affinity receptor activated at low concentrations, which stimulates DNA synthesis and cellular proliferation; and the low-affinity/high-capacity receptor becoming dominant at higher concentrations decreasing the cell proliferative response. This is a general pharmacological mechanism in that it is employed for a large number of receptor-based responses from cancer cells to neutrophil chemotaxis and many others.

This article reviews hormetic effects of various agents on skin biology. Recognition of this emerging biological phenomenon in dermatology should lead to markedly improved integrative assessments of animal/human skin responses to toxic substances, pharmacological agents, and endogenous agonists.

87.2 EVIDENCES OF HORMESIS IN SKIN

Skin is a complex biological model but highly approachable. Models exist for dermatological research, which include animal versus human skin models, *in vitro* versus *in vivo* models, regional variation, stem cell biology, and hair follicle biology. Many pharmaceutical preparations in dermatology affect cell regulation. Nonetheless, the FDA sometimes exempts dose justification for dermatologic preparations. As a result, the presence of any hormetic effect might have been missed.

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TABLE 87.1
Examples of Hormesis in Skin

Chemicals	Cell Type	Stimulatory Responses	Inhibitory Responses	References
Sodium lauryl sulfate (SLS)	Cultured k eratinocytes	SLS (10^{-8} to 10^{-5} M): For 1 h: 36% stimulation For 18 h: 12% stimulation For 4 days: ~89% stimulation	$>10^{-5}$ M	[7]
	Cultured fibroblasts	Subconfluent fibroblast: For 1 h (10^{-5} M): 38% stimulation For 18 h (10^{-6} M): 32% stimulation Confluent fibroblast for 1 h (10^{-8} M): 40% stimulation		
SLS versus retinoic acid (RA)	Cultured keratinocytes	0.1–1 $\times$ 10^{-5} M: 1. For 2 h: from 4 $\times$ 10^{-4} (baseline cell count) to a maximum of 8 $\times$ 10^{-4} cells 2. Less potent than RA at 0.75–3 $\times$ 10^{-6} M	$>5 \times 10^{-5}$ M	[8]
	Cultured dermal fibroblasts	1. Similar stimulatory response as keratinocytes at the same doses 2. Less effective than RA in stimulating production of extracellular matrix		
Imidazole derivatives (econazole, clotrimazole)	Reconstructed human epidermis	5–133 μM (18 h contact time): Biphasic effect on 7-ethoxycoumarin-*o*-deethylase (ECOD) activity in the epidermis, with induction (60–70% increase of the basal level) at low concentrations and inhibition (40% of the basal level) at high concentrations	>133 μM	[9]
Ionizing radiation	Cultured human dermal fibroblasts	The plating efficacy reached values significantly above 100% apparent survival at low doses (≤40 cGy)	≥40 cGy	[10]
Corticotropin-releasing hormone	Cultured human sebocytes	Biphasic effect on sebaceous lipid synthesis and upregulation on the mRNA levels of 3-β-hydroxysteroid dehydrogenase/$\Delta^{5\text{-}4}$ isomerase (10^{-7} M), but did not affect cell viability, cell proliferation, or interleukin-1β-induced interleukin-8 release		[11]
Arsenite	Human epidermal keratinocytes, promyelocytic leukemia cells	Exposed to arsenite from 0.1 to 40 μM for 1, 3, and 5 days, cell growth was increased at low doses (0.5 μM)	>1 μM	[12]
Arsenic trioxide (As)	Keratinocytes, melanocytes, dendritic cells, dermal fibroblasts, microvascular endothelial cells, monocytes, T cells	Exposed to for 72 h: 1. Sublethal doses of As stimulate cell proliferations 2. As is toxic at high doses to keratinocytes, fibroblasts, monocytes, and T cells; and toxic at low doses to melanocytes, microvascular endothelial cells, and dendritic cells Peak proliferation: Keratinocytes: 30 μM; melanocytes: 0.95 μM; dendritic cells: 0.96 μM; fibroblasts: 7.6 μM; microvascular endothelial cells: 0.95 μM; monocytes: 30 μM	Lethal dose (LD_{50}) LD_{50}: Keratinocytes: 45.5 μM; melanocytes: 7.6 μM; dendritic cells: 7.6 μM; fibroblasts: 187 μM; microvascular endothelial cells: 2.4 μM; monocytes: 252.7 μM	[13]
Mixture of four metals (arsenic, chromium, cadmium, lead)	Human keratinocytes	Exposed for 24 h: Lowest mixture dilution (0.0014× of As 7.7 μM, Cr 4.9 μM, Cd 6.1 μM, Pb 100 μM)) with a total concentration of 0.163 μM had a percent viability of 116.6%, clearly above that observed in the single-chemical data. It is likely that this enhancement of cell viability at the lowest mixture level is indicative of the presence of hormesis	Synergistic cytotoxicity at total concentration of 8–36 μM of the metal mixture	[14]
Arsenite (iAs^{III}), arsenate (iAs^{V}), methylarsine oxide ($MAs^{III}O$), complex of dimethylarsinous acid with glutathione ($DMAs^{III}GS$), methylarsonic acid (MAs^{V}), dimethylarsinic acid ($DMAs^{V}$)	Human keratinocytes	1. iAs^{III} and $DMAs^{III}GS$ induced an increase in cell proliferation at low concentrations (0.001–0.01 μM), while at high concentrations cell proliferation was inhibited 2. Pentavalent arsenicals did not stimulate cell proliferation 3. Methylated forms of As^{V} were more cytotoxic than iAs^{V}	iAs^{III}: >0.5 μM; iAs^{V}: >1.0 μM; $DMAs^{III}GS$: >0.05 μM; $DMAs^{V}$: >0.1 μM	[15]

TABLE 87.1 (continued)
Examples of Hormesis in Skin

Chemicals	Cell Type	Stimulatory Responses	Inhibitory Responses	References
Minoxidil	Human epidermal and follicular keratinocytes	0.1–10 μM (exposed for 5–8 days): Minoxidil had biphasic effects on the proliferation and differentiation of both epidermal and follicular keratinocytes, stimulating proliferation at micromolar doses, while antiproliferative, prodifferentiative, and partially cytotoxic effects were observed with millimolar concentrations	>1 mM	[16]
Nitric oxide (NO 3-morpholinosydnonimine) donors: Nitroprusside, SIN-1, diethylenetriamine DETA/NO, SNAP S-nitroso-N-acetylpenicillamine	Human keratinocytes, fibroblasts	Four different NO donors at concentrations ranging from 0.01 to 5 mM were added every 12 or 24 h, and cells cultured for up to 3 days in the presence of these compounds. Keratinocytes: 1. A biphasic effect is found with increased proliferation at low concentrations and cytostasis at high concentrations 2. Cytokeratin 6 expression is decreased at the lower NO donor concentrations and increased at higher concentrations as an indication of induction of differentiation at higher NO concentrations Fibroblasts: Cytostasis becomes significant at ≥0.25 M of the NO donor		[17]
Antioxidants: Ascorbic-2-*O*-phosphate (Asc2P), ascorbic-2-*O*-alpha-glucoside (Asc2G)	Human keratinocytes	Repetitive addition of Asc2P and Asc2G: Cellular life-span of keratinocytes was shown to be extended up to 150% of population doubling levels (PDLs)		[18]
Prooxidants: Hydrogen peroxide (H_2O_2)		Prooxidants: 20 μM H_2O_2: extended up to 160% of PDLs 60 μM H_2O_2: extended up to 120% of PDL		
1,25-Dihydroxy vitamin D_3 ($1,25(OH)_2D_3$)	Whole organ cultures of hair follicle	Biphasic dose–response relationships for the effects of $1,25(OH)_2D_3$ on the total cumulative growth of hair follicles and hair fibers At relatively low concentration, growth of follicles and fibers was stimulated, to a maximal extent at 10 nM of 52 and 36% The concentration producing 50% of the maximal response (EC_{50}) for both follicle and fiber growth stimulation was 0.3 nM The increase in cumulative growth was due to stimulation of the initial, linear growth phases	Dose-dependent and complete inhibition of follicle and fiber growth at 100 nM	[19]
Ciprofloxacin (CPFX)	Human fibroblasts	The effect of CPFX on cell viability is time dependent: 1. CPFX was not cytotoxic at any concentration when the cells were incubated for 24 h 2. Low concentrations (0.0129 and 0.032 mM) of CPFX increased the cell survival in all incubation periods tested	Decreased viability was observed at 0.129 and 0.194 mM (48 h of exposure), and 0.129 M (72 h of exposure)	[20]

The literature in dermatology indicates that several cell types in the skin provided evidence of hormetic-like biphasic dose/concentration–response relationships. A brief listing of the cell types showing hormetic relationships and the quantitative features of dose responses is presented in Table 87.1.

87.3 MELANOMA AND TUMOR CELL LINES DISPLAY HORMETIC DOSE RESPONSES

Perhaps a more important issue regarding hormesis is its relationship to cancer biology. The existence of hormetic dose responses in many tumor cell lines has been noted and reviewed

TABLE 87.2
Examples of Dermatology-Relevant Chemicals Displaying Hormetic Dose–Response Relationship in Tumor Cell Lines

Chemicals	Tumor Cell Lines	Possible Mechanisms	References
Endogenous agonists			
Epidermal growth factor (EGF)	Ovarian, colon, epidermoid, breast	A431 cells: 1. The dual effect (stimulation/inhibition) of ECF on its proliferation is associated with differential pattern of mitogen-activated protein (MAP) kinase activities, which may involve the action of specific phosphatase(s) 2. Dependent on the quantity of occupied EGF-R: A critical and restricted number of sites are involved in EGF-growth stimulation 3. Low-dose stimulation is mediated by a minority population of high-affinity EGF-Rs	[21–24]
Estrogen	Colon, breast	Colon cancer cells: Physiological concentrations of estradiol acting via the classical Estrogen receptor (ER) may have a proliferative effect. When there are high luminal concentrations of estrogenic compounds, they may act on low-affinity estrogen binding sites that mediate the growth-inhibitory effect	[25]
Progesterone	Ovarian	HOSE and Oca cells: Stimulation by progesterone at low concentrations, marked inhibition at high concentrations, both blocked by specific progesterone antagonist, confirming the specificity of the hormonal action	[26]
Phytocompounds			
Daidzein	Breast	1. Isoflavones elicit a biphasic response in the DNA synthesis and cell proliferation of the ER of positive human breast cancer cells 2. Effects of diadzein and biochanin A on these cells appeared to be associated with the expression of P53	[27]
Genistein	Colon, breast, oral	1. Binds to the ER at estrogen binding site; the formed complex then interacts with the estrogen response element ERE1 thereby promoting the transcription of estrogen-regulated genes 2. MCF-7 cells: Cell proliferative effects were mediated through ER, while antiproliferative effect was independent of ER	[28,29]
Glabridin	Breast	Proliferation of ER^+ cells was highly associated with the binding affinity of glabridin to the ER. Optimal cell proliferation occurred at a concentration at which half of the ER sites were saturated	[30]
Quercetin	Breast, oral	1. Similar to genistein, a biphasic effect on cell proliferation with ER involvement 2. Regulatory over corrections by biosynthetic control mechanisms to low levels of growth inhibiting challenge 3. Concentration-dependent antioxidant and prooxidant activities	[31]
Resveratrol	Breast, leukemia	1. MCF-7 cells: At low concentration, acts as a partial ER agonist. At high concentrations, causes inhibition of MCF-7 cells regardless of ER status, possibly via the antagonizing of linoleic acid (a potent stimulator of breast cancer cells)	[32]
Drugs			
Dexamethasone	Neuroepithelial, pancreas, meningiomas	1. Brain tumor: Low-dose stimulation is related to the presence of glucocorticoid receptor (probably necessary but insufficient). Inhibitory effects at high doses were believed not to be due to receptor mediation but by other mechanisms such as cell membrane alterations 2. Neuroepithelial cancer cells: a. Dexamethasone treatment causes glucocorticoid receptors translocation into the nucleus to modulate cell proliferation upon binding of different concentrations of dexamethasone. Dexamethasone inhibits proliferation of some neuroepithelial cell lines, not by glucocorticoid-induced apoptosis b. Lower concentrations of dexamethosone stimulate growth only in glucocorticoid-positive tumors, suggested the role of the specific receptor. Higher concentrations inhibit cell growth not due to receptor mediation, but seem to be related to other mechanisms (cell membrane alterations)	[33–35]
Retinoic acid	Breast, prostate, gliobastoma	1. Breast MCF-7 cells: via insulin-like growth factor-1 (IGF-1) receptor: Lowering IG7-1 levels inhibits cell proliferation 2. Prostate LNCaP cells: Possible roles of retinal-binding proteins and retinoic receptors, which may have biphasic mitogenic effects on LNCaP cells and are concentration dependent in affecting prostate-specific antigen (PSA) secretion	[36,37]

TABLE 87.2 (continued)
Examples of Dermatology-Relevant Chemicals Displaying Hormetic Dose–Response Relationship in Tumor Cell Lines

Chemicals	Tumor Cell Lines	Possible Mechanisms	References
Toxic substances			
Cadmium chloride	Ovarian	No cytotoxicity at low concentration, but has stimulatory effects on metabolic activities particularly in mitochondria via unknown mechanism	[38]
Sodium butyrate	Colon	Dependent on the other energy sources available to epithelium: In conditions of low energy availability, butyrate could be both stimulatory/trophic. In the presence of high levels of alternative energy sources such as glucose, butyrate could inhibit growth/induce apoptosis	[39]

by Calabrese [4]. Twelve melanoma cell lines (M4Beu, B16, M24, MNT, SK-MEL, H1144, SK-MEL28, Cal 1, Cal 4, Cal 23, Cal 24, and Cal 32) have been shown to display hormetic dose responses to various chemicals (guanine or guanosine derivatives, mistletoe extract, salsolinol, tetrahydropapaveroline, dopamine, resveratrol, thrombin, and suramin). Numerous endogenous agonists, drugs, environmental contaminants, and phytochemicals, some relevant to dermatotoxicology and dermatooncology, have also been noted to exert hormetic dose responses in various tumor cell lines [4]. Examples and the proposed mechanistic explanations are listed in Table 87.2.

87.4 DISCUSSION

Calabrese and Blain [40] developed a hormesis database, containing 5600 hormetic-like dose–response relationships over approximately 900 agents from a broadly diversified spectrum of chemical classes and physical agents, stressing the general robustness of published studies to establish support for the hormetic dose–response hypothesis. Table 87.1 showed that clear examples of hormesis do exist in dermatology, and Table 87.2 suggested that the presence of hormesis in cancer biology may be an important phenomenon not to be overlooked.

Despite the extensive observation of hormetic dose–response relationships for numerous agents across the biological spectrum, most studies assessed cellular responses. Few studies followed up in animal and human models—normal or diseased— assessed the simultaneous responses of different systems to the same agent. We believe *in vivo* studies are necessary to provide an integrative assessment of the whole animal/human responses to various agents, to document any discrepancies between the *in vitro* and *in vivo* responses, and to clarify the clinical implication of hormesis.

Studies on the mechanism of action and the exact definition of the low dose to be applied are essential to achieve a better understanding of hormesis. Another important issue to discuss in the field of hormesis, as proposed by van der Woude et al. [41], is the need for risk assessment paradigms to be modified to take hormesis into account. Rietjens and Alink [42] also suggested that more focus should be redirected from looking only at adverse effects at high levels of exposure to characterize the complex biological effects, both adverse and beneficial, at low levels of exposure. Low-dose toxicology and pharmacology will contribute to better methods for low-dose risk assessment of chemical compounds and their effect on carcinogenesis, taking into consideration that the ultimate biological effect of a chemical may vary with its dose, the endpoint or target organ considered, cellular interactions, and the combined exposure with other chemicals.

TABLE 87.3
Targets for DNA Microarray Studies in Dermatology and Skin Biology [45]

Melanoma and melanocytes
Carcinomas (basal cell carcinoma, squamous cell carcinoma)
Keratinocyte differentiation
Wound healing and inflammatory diseases
Proinflammatory and immunomodulating cytokines in skin
Effects of ultraviolet radiation (UV) and environmental stress
Epidermal stem cells and the hair cycle
Fibroblasts and other cutaneous cell types
Artificial skin substitutes

We believe skin is an excellent candidate to gain entrance into this biology due to its accessibility; its complex nature, with highly differentiated cell types and various subsystems (keratinocytes, melanocytes, Langerhans cells, fibroblasts, epidermis, dermis, hair follicle, eccrine, apocrine, and sebaceous units); and the availability of specialized noninvasive technology for *in vivo* studies [43,44]. In addition, skin has been among the first organs analyzed using DNA microarrays in various topics from skin cancers, melanomas, basal cell carcinomas, squamous cell carcinomas, psoriasis, and other inflammatory disorders, to stem cell biology, the biology of epidermal keratinocytes, and so forth (Table 87.3) [45]. DNA microarray studies will be an excellent tool to elucidate the mechanisms of hormesis in skin biology. In short, better understanding of hormesis will likely lead to different strategies for risk assessment process employed in the fields of dermatologic toxicology and pharmacology.

87.5 CONCLUSION

Hormesis is a common phenomenon in dermatology and other fields. Detailed consideration should be given to its concept, its risk assessment implications, and its clinical significance. However, without additional mechanistic insight,

the consequences of hormesis for risk assessment and the possibilities for *in vitro* to *in vivo* extrapolation will remain limited.

Skin can be an excellent candidate to study hormesis and its underlying mechanisms because of its accessibility; its repertoire of inflammatory and immunomodulating cytokines, hormones, vitamins, unique responses to ultraviolet light, toxins, and physical injury; and the availability of noninvasive bioengineering and DNA microarray technology. Artificial skin substitutes are also available to study the effects of harmful or dangerous agents. In essence, the skin has everything: from stem cells, signaling, and cellular differentiation, to inflammation, diseases, and cancer. All these facets could become excellent models to further study hormesis and its clinical implications following exposure to a variety of toxic compounds and pharmaceutical agents.

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88 Diagnosis of Drug Hypersensitivity *In Vitro*

Hans F. Merk and D. Hoeller Obrigkeit

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88.1 INTRODUCTION

Drug-induced skin rashes occur in 2–3% of hospitalized patients. Depending on the pharmacological properties of a drug, reactions can be rated as an A-reaction (expected reaction) or B-reaction (unexpected or bizarre reaction). A-reactions comprise toxicity, for example, overdosage, inevitable side effects at a dosage necessary to achieve the desired effect such as chemotherapeutic agents as well as drug interactions. B-reactions result from the specific properties of each active pharmaceutical ingredient (API) and individual risk factors of the patient. A major cause of B-reactions is allergic reactions, which requires a specific T-cell-dependent sensitization of the patient leading to T-cell- or antibody-dependent allergic reactions. The skin is a preferred target organ for B-type reactions. Dangerous reactions include anaphylaxis and angioedema as well as bullous drug reactions, especially toxic epidermal necrolysis (TEN). Most often involved drugs are antibiotics—in particular β-lactam antibiotics, sulfonamides, and quinolones—anticonvulsa nts, contrast media, and cytotoxic agents such as cisplatin derivatives. Investigations about the function of basophiles and their activities as well as the function of T cells in delayed-type reactions improved our understanding of the pathophysiology and led to new diagnostic options including *in vitro* assays. Recently established animal models as well as investigations on the level of antigen-presenting cells may improve the ability to predict the immunogenicity of low-molecular-weight compounds.

The aim of diagnostics in allergic and pseudoallergic drug reactions is to identify the causing API and, if possible, the mechanism of action causing the disease. Especially the differentiation of allergic and pseudoallergic reactions is important for risk assessment of future exposure with the drug [5]. These diagnostic procedures include an accurate history and *in vivo* tests such as patch tests, prick, and intracutaneous test, and may also involve in selected cases a graduated challenge at least to exclude the allergic reaction to a particular drug. A further possibility is *in vivo* tests. Most important is that the patient should receive an allergy card at the end of every allergy diagnostic. The card should indicate not only the suspected API, but also the basis of diagnostics (patient history, specific IgE, positive skin test reaction, or re-exposure) and, if checked, an alternative API to use.

In vitro tests have the advantage of not endangering the patient. In addition, pathophysiological factors of a drug reaction might be revealed by these tests. There are serological and cell-based tests available (Table 88.1). Serological tests are usually performed at central allergy units or laboratories, and are easy to evaluate in epidemiological studies. Cell-based tests require the presence of a patient at the testing site or elaborate logistics to ensure fast processing of blood or tissue samples.

88.2 SEROLOGICAL TESTING SYSTEMS

The most important serological test is the determination of specific IgE after anaphylaxis. The RAST (radioallergosorbent test) is used for this investigation. It is a solid-phase, radio-immunoassay that measures circulating allergen specific IgE antibody. This test as well as analogs procedure, which has a different solid-phase or a different end point measurement, is performed by linking the drug in question to a solid phase. This is incubated with the serum of the patient, during which time specific antibodies—including IgE—are bound. After washing, a second incubation is done with a highly specific anti-IgE-antibody and the amount

TABLE 88.1
***In Vitro* Assays in Drug Allergic Reactions**

I. Serologic assays
Specific IgE (β-lactam antibiotics)
Tryptase
II. Cellular assays
Basophil activation (histamine release, sulfoleukotriene release, CD63 expression)
Lymphocyte transformation assay

of this binding is measured afterwards. Routinely this is performed in penicillin sensitization only. Specific IgE to latex or muscle relaxants can be measured if the patient reports a specific history during general anesthesia or other procedures in which latex may have been used. Propositions to identify specific IgE to acetylsalicylic acid, codeine, phenazon, tartrazin, or contrast agents are not subject of reliable allergy diagnostics because results from a serum under study should be compared with a positive reference serum and negative control serum. This has been done besides reactions to β-lactam antibiotics in the case of allergic reactions to quinolones [25]. In this study, the test was performed in 55 patients with immediate-type adverse reactions to quinolones and in 30 (54.5%) patients this test yielded positive results when the test was performed 1–48 months after the reaction. Interestingly there was no increase of atopic patients who developed IgE antibodies against quinolones but the IgE seemed to disappear more slowly in atopic patients [25].

In addition, measurements of tryptase levels in serum up to 2 h after severe anaphylaxis are used to diagnose basophilic lymphocyte involvement in the reaction. Thus, anaphylaxis can be confirmed. Furthermore, the constant elevation of this value without a recent anaphylactic shock indicates a mastocytosis that might explain the severity of the allergic reaction [41].

88.3 CELLULAR TESTING SYSTEMS

Cell-based allergy diagnostics are performed with basophilic leukocytes and T lymphocytes.

88.3.1 Basophil Leukocyte Test

This test determines the reactivity of basophil leukocytes after incubation with the suspected drug. Reactions are evaluated by measurement of released histamine (histamine-release test), the released cysteinyl leukotrienes (CAST-ELISA). The sensitivity of the CAST-ELISA is enhanced by the coincubation of the basophiles with interleukin 3. Another method is the fluorescent detection by flow cytometry of CD63 in activated basophilic leukocytes. This assay has lately been described as a promising tool in the diagnostic of allergies to muscle relaxants and β-lactam antibodies. In combination with a CAST-ELISA, the sensitivity of diagnostics in analgesic intolerance could be improved [11]. In a recent multi-center European study which included 181 patients, a sensitivity of the diagnosis in β-lactam antibiotic allergic reaction of 94.6% could be achieved if one combined skin test, the determination of specific IgE, and the CAST-COMBI test were performed. In this assay, the flow cytometric determination of basophil activation (CD63 expression) and sulfoleukotriene production (ELISA) after preincubation with the drug and IL3 are performed. The specificity was 75% [48]. Data of the same group showed the possibility to use the Flow-CAST assay in patients with NSAID hypersensitivity. This study demonstrated that in this case a precise dose–response curve is required [49].

88.3.2 Lymphocyte Transformation Test (LTT)

In vitro tests of basophilic leukocytes only detect immediate-type allergic or pseudoallergic reaction. T lymphocytes, on the other hand, play a central role in immediate as well as late-type immune response. The lymphocyte transformation test (LTT) has been widely employed to identify T-cell-related responses to drugs *in vitro* in sensitized patients. In short, lymphocytes of sensitized patients and healthy controls are incubated with the drug in question. Drug-specific T-cell proliferation is measured after 5–7 days by H^3-thymidine marking of the cells. In the past, the LTT has been helpful in individual cases to objectify T-cell reactions to an API, for example, β-lactam antibiotics, anticonvulsives, or sulfonamides [26,33]. In most cases, contrast media elicit an intolerant reaction by histamine release. However, single patients, especially after late-type reactions, show a positive reaction in patch test and LTT, suggesting an allergic reaction in these patients [23]. Usually, late-type reactions are not severe or life-threatening but manifest as itching with or without urticaria and only rarely show a more severe progression.

A high quality of results requires meticulous planning, including the controls. It is recommended to perform an LTT within 3 months of the allergic reaction. Due to a low frequency of drug-specific T lymphocytes in peripheral blood, the measurable drug-induced T-cell proliferation is low. This complicates the differentiation of significant positive and negative results [19]. These findings are supported by Roujeau et al. [38]. Their study revealed that a few months after a TEN, no drug-specific T-cell responses could be determined, although in isolated cases T-cell-specific reactions to APIs could be determined for years [2]. Despite these difficulties, the LTT is more specific and sensitive towards allergic reactions to β-lactam antibiotics and local anesthetics, than skin tests. In selected cases, this elaborate test might be expedient and should be performed in experienced laboratories.

Modifications of this test have recently been introduced:

1. Measurement of the expression and release of cytokines such as IFN-γ, IL-5 as an end point instead of T-cell proliferation
2. Isolation and cloning of lesional T lymphocytes
3. Incubation not only with the parent drug but also with its metabolites or drug-metabolizing systems [26,42–44]

Not only proliferation is measured as an end point, but also the expression of surface activation markers such as CD69 or antigen-specific cytokine profiles are being assayed [14,42,43]. In our own studies, drug-specific activation of peripheral blood mononuclear cells consistently resulted in IL-5 and to a lesser extent in IL-10 and IFN-γ concentrations by ELISA secretion. This secretion was heralded by an earlier mRNA expression of these cytokines, which were measured by RT-PCR [42]. Also the expression and release of IFN-γ was used as an end point determination in the LTT [14,15].

Further on, lesional T lymphocytes and specifically sensitized T-cell clones were obtained from cutaneous drug reactions [18] to study the pathophysiology of allergic drug reactions. In case of penicillin-induced bullous drug reaction only CD8+ T cells could be isolated from epidermal skin lesions [16]. Similarly, mainly CD8+ T cells could be isolated from test reaction areas of patients after epicutaneous application of penicillin [18]. These isolated CD8+ T cells produce cytokines including IL-2 and IFN-γ, but not IL-4 [27]. Similar observations were made in epidermal CD8+ T-cell clones in patients suffering a morbilliform exanthema to penicillin [6].

In addition, T lymphocytes cloned from lesions that reacted antigen specific to drugs, such as to penicillin, revealed cytotoxic qualities in classic immunological assays against B lymphocytes. Cytotoxic reactions were also seen in target cell, in the case of a TEN in keratinocytes [18]. Further studies could confirm cytotoxic qualities of epidermal T lymphocytes, but reactions differed in bullous skin reactions, drug exanthema, and AGEP. Thus, in drug exanthema and AGEP CD4+ T cells dominated besides CD8+cells. In TEN, CD8+ cells were the dominant cell type [24]. In drug exanthema, h igh level of IL-5 could be detected, while in bullous reaction IFN-γ and in AGEP IL-8 is overexpressed [4,24]. Cloning of T lymphocytes isolated from AGEP lesions revealed the presence of cytotoxic T lymphocytes in this disease. Furthermore, an increase in IL-8 expression could be detected in the lesions. This is one explanation for the diagnostic difficulty in distinguishing AGEP from psoriasis [4].

88.4 ROLE OF DRUG METABOLISM

In contrast to the above-mentioned examples of β-lactam antibiotics, most drugs are metabolized by the body. In the first phase of metabolization, oxidative reactions change an API into a highly reactive chemical substance with a high affinity to proteins and peptides. These reactions are mainly mediated through cytochrome P450 isoenzymes that are primarily located in the liver but are also present in extra-hepatic organs, including skin. [1]. This metabolic pathway emphasizes the postulation by Landsteiner: low-molecular-weight haptenes cause sensitizations by binding to peptides or proteins. Analysis of anticonvulsives—phenytoin, carbamazepine, and lamotrigen—and sulfonamides generated evidence for the importance of oxidative metabolism in developing an API sensitization. Important enzymes include the above-mentioned cytochrome P450 isoenzymes. The incubation of keratinocytes with metabolites of sulfonamides, which are generated from CYP-dependent metabolic pathways are bound to membrane proteins in opposite to the parent compound [39,40].

Anticonvulsives such as phenytoin, carbamazepine, lamotrigen, or benzodiazepam are metabolized to highly reactive arenoxide metabolites by cytochrome P450-dependent enzymes and can be detoxified by epoxide hydrolases. Alternatively, cytotoxic intermediate metabolites can bind to a protein and therefore can be immunogenic [45]. A lymphocyte toxicity assay has been developed to help determine a phenytoin sensitization *in vitro*. In addition, the lymphocyte reaction to phenytoin and carbamazepine could be improved after addition of liver microsomes with cytochrome P450 activity to LTT [12,13]. It was also shown that skin expresses cytochrome P450 isoenzymes capable of metabolizing carbamazepine into reactive metabolites that bind covalently to proteins [50]. Finally, antibodies to cytochrome P450 3A4, the enzyme metabolizing anticonvulsives, could be detected in patients with sensitization to anticonvulsives. T-cell clones of these sensitized patients reacted to metabolites of carbamazepine [9,10,36]. The role of such antibodies in diagnostics needs further evaluation.

Benzodiazepam allergies are characterized by a cytochrome P450-dependent sensitization as well as an increased T-lymphocyte proliferation and an increased IL-5 expression and release [43]. These findings are in accord with findings in phenytoin sensitization. In these cases, a polyclonal stimulation was detected in LTT; on a molecular level, cell activation required processing of phenytoin [8].

Sulfamethoxazole (SMX) is acetylated to N4-acetyl-SMX and metabolized by cytochrome P450 2C6 to 5-hydroxy-SMX or a reactive hydroxylamine [10]. The hydroxylamine metabolite can be eliminated by glutathione synthetase or, after spontaneous oxidation, formed into nitroso-SMX that becomes immunogenic by binding to macromolecules. Our own findings with SMX-specific T cells in a patient with a bullous exanthema revealed a significant proliferation of SMX and autologous irradiated lymphocytes as antigen-presenting cells *in vitro*. Even after addition of SMX-modified murine liver microsomes that were rich in drug metabolizing cytochrome P450 enzymes, T cells proliferated. The induction was shown despite the fact that oxidized sulfonamides and their metabolites revealed an immunosuppressive activity in T cells [17,20,21,22]. Glucose-regulating protein 78 (grp78) and the proteindisulfide isomerase could be identified as the binding structure in the endoplasmic reticulum [36]. Although these studies did not reveal the pathophysiological role of these antibodies as cause or epiphenomenon of the reaction, they underline the importance of the oxidative, cytochrome P450-dependent metabolism in sensitization of a patient.

The antiviral drugs abacavir and nevirapine cause drug exanthema or bullous drug eruptions in up to 10% of the patients. Patients reacting to abacavir had a strong association to the allele HLA-B5701; patients who reacted to nevirapine were correlated to HLA-DRB1*0101 and a comparably high CD4+T-cell fraction. The reaction to abacavir can be detected

by a patch test and shows a 100% correlation to HLA-B*5701 [31]. To diagnose sensitizations to nevirapine, an animal model has been developed [7,35]. The model revealed that the sensitization was primarily induced by a cytochrome P450-dependent 12-hydroxy-metabolite of nevirapine. Interestingly, the API, but not the metabolite, was cognized by T-cell clones that could be isolated after the sensitization. This demonstrates that a sensitization might be caused by metabolites of an API but not recognized by T lymphocytes or vice versa: APIs that are recognized by sensitized T cells do not necessarily reveal the conditions leading to the sensitization [35].

Studies on T-cell clones isolated from patients sensitized to an API revealed that the API in question was able to activate sensitized T lymphocytes directly. These results refute earlier findings that oxidative metabolism of an API is necessary to develop antigens. This type of reaction is found in sensitization to local anesthetics, anticonvulsives, sulfonamides and, in our own studies, in contact allergies such as *p*-phenylenediamine [33,47]. These observations suggested the i.p. principle—a direct T-lymphocyte reaction to such substances without prior processing by an antigen-presenting cell [32,33]. One has to bear in mind that most T-cell clones examined reacted to the API as well as to its metabolite(s). In the murine system, sensitizations were only inducible with oxidized metabolites [22,28,29]. Furthermore, especially the model for nevirapine sensitization showed that metabolites other than those detected by T cells might play a role. These findings underline that the i.p. principle does not sufficiently explain antigen processing and presentation in the primary immune response. Metabolization seems to be at least a necessary danger signal in this phase of sensitization. Another hypothesis of the i.p. principle is that drugs attached to other substances, for example, peptides, are identified by chance. This assumption cannot be supported by any data at this time and is subject to speculation. Despite these drawbacks, the i.p. principle can still be applied to LTT diagnostics (secondary immune response) as demonstrated for sulfonamides or *p*-phenylenediamines in contact eczema [47].

88.5 OUTLOOK

The influence of the maturation of antigen-presenting dendritic cells on the pathophysiology of adverse cutaneous drug reactions to β-lactam antibiotics was studied recently [37]. Amoxicillin drove dendritic cells from hypersensitive patients to a phenotypic and functional semimature status, inducing a T-cell proliferation response [37]. In addition, the characterization of low-molecular-weight compounds as sensitizers under *in vitro* conditions with human immunocompetent cells could be shown by the incubation of such compounds with xenobiotic-metabolizing organ-specific CYP-cocktails and the xenobiotics in question with human dendritic cells [3]. These results indicate the possibility to identify high-risk compounds or the possibility to establish a risk–benefit ratio before such compounds are used in humans. Also animal models of allergic drug reactions were established [30,46]. On the other side individual risk factors for allergic reactions to xenobiotics are identified such as polymorphisms for cytokines or cytokine receptors or specific HLA-alleles, which may predict patients with an increased risk [34]. These approaches might be able not only to diagnose drug allergic reactions but also to prevent them.

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89 Immunologic Patterns in Allergic and Irritant Contact Dermatitis: Similarities

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89.1 CONTACT DERMATITIS

Contact dermatitis (CD), an inflammatory response in the skin due to external stimuli, is a complex phenomenon involving epidermal cells, fibroblasts, and endothelial cells as well as invading leukocytes interacting under the control of a network of cytokines and lipid mediators [1,2]. Traditionally divided into irritant and allergic mechanisms, the former results from toxic irritant chemicals and the latter represents a delayed-type hypersensitivity reaction [3]. Irritant contact dermatitis (ICD) and allergic contact dermatitis (ACD) share similar cellular and molecular pathways to achieve the same compensatory outcome, the reestablishment of dermal homeostasis [4].

The physiologic events in its development involve penetration of the corneal barrier of irritant or allergen, interaction with epidermal or dermal cells, interaction with the immune system, and inflammatory response [5].

The basis for a diagnosis of either is determined by combining morphology, histology, and immunologic findings in relation to its time course [6]. However, new information provided by studies attempting to define a distinction between immunologic and nonimmunologic events, in both conditions, makes differentiation more complex [7].

Mathias outlined the factors influencing response to irritants, proposing that only in extreme examples of irritation, are events that distinguish irritant from allergic reactions overt [7].

The immunopathologic differences between allergic and irritant reactions remain unresolved. Many believe that ACD and ICD cannot be differentiated by morphologic, histologic, or electron microscopic examinations. The demonstration of a type IV immune reaction remains a specific point of difference [5]. In general, most studies reveal similarities rather than clear-cut differences between ACD and ICD. These similarities concern both the localization and cellular composition of the skin infiltrates [8]; the expression of activation markers expressed by keratinocytes, such as intercellular adhesion molecule (ICAM)-1 and human leukocyte antigen DR (HLA-DR) [9,10]; and cytokine profiles [11,12]. Owing to conflicting data on the nature of the inflammatory responses in ACD and ICD, there is currently no single marker to distinguish ACD and ICD [13].

Many studies attempted to differentiate ICD and ACD; none were overtly successful. Clearly defining differences between irritant and allergic dermatitis have been moot. Table 89.1 lists some issues requiring factorial analysis before this intellectual battle can be declared over.

89.1.1 Irritant Contact Dermatitis

ICD is defined as chemically induced dermatitis that is nonimmunologic—a heterogenous disease caused by different chemicals. Its etiopathology is incompletely understood. Histologically, it can often be difficult to differentiate between ACD and ICD. Although induced differently, ACD and ICD are similar clinically and histologically. ICD itself differs between acute and chronic phases and the pattern of damage differs according to the specific irritant [14,15].

ICD was previously considered as a simple toxic damage to the cutaneous cell system, but in truth, it presents a

TABLE 89.1

Physical chemistry of allergens and irritants	All may not be handled similarly
Time course observations	Limited data suggest complex processing
Species differences	Human validation eventually required
Multiple biochemical endpoints	See text for details

complex reaction pattern. Many studies suggest a major role of the immune cells regulating this process.

The pathologic mechanisms of ICD remain incompletely defined. It is not clear how irritants applied to the skin cause inflammation [16]. When provocative substances penetrate the stratum corneum, it seems probable that immune surveillance mechanisms must be activated. In irritant reactions many provoking substances act like haptens or may modify self-peptides so that their combination with class II major histocompatibility complex (MHC) determinants will be recognized by antigen-specific elements within the normal mature CD4+ helper T cell repertoire [17,18].

The most unknown aspect of ICD is the endogenous modulation of response. The pathways that lead to inflammation and the reason why subjects develop different degree of irritation when exposed to the same irritant (test compound) under the same conditions remain sub judice [19].

To assess whether cytokines contribute differentially over time during the course of an inflammatory response, we reviewed the role of proteins released during induced contact reactions in ICD and ACD and the similarities, possibly immunologic common aspects between both entities.

89.1.1.1 An Immunologic Component in ICD

ICD and ACD may show a remarkable similarity with respect to morphology, histology, and immunohistology [13–15, 20,21]. Studies meticulously compare the pathogeneses of the mechanisms involved in the inflammatory process of both.

Brasch et al. [9] showed that antigen recognition by specific memory T cells as well as irritants can induce the same pattern of inflammation, including activation of T cells independent of exogenous antigen.

Many irritants initially act via a common mechanism [22]: a diversity of unrelated chemicals, when applied in suitable concentrations, results in moderate skin reactions that share characteristics such as mild damage to keratinocytes, a predominantly mononuclear dermal infiltrate, and apposition of lymphocytes with Langerhans cells (LC) [23,24].

Three patterns of inflammatory cells appear essentially identical in both, consisting of T lymphocytes of the helper/inducer type mainly in association with LC- and HLA-DR-positive dermal macrophages [8,13,21,25–29].

Brand et al. described that immunocompetent cells migrating from the area of CD to the regional lymph nodes, together with increased functional capacities of the lymph cells [30], and augmented output of soluble mediators such as cytokines [31] and complement proteins [32], indicate immunologic activity [33].

Recent studies failed to demonstrate significant differences between ICD and ACD [13,34,35] because the patterns of T-cell infiltration and cytokine release are similar.

Many studies correlate the role of the applied compounds and their evidence in stimulating cutaneous reactions induced by allergens and irritants. A reproducible pattern of cytokines released including interleukin-1 (IL-1) and tumor necrosis factor-alpha (TNF-α) molecules is seen in both ACD and ICD [36,37]. Nickoloff [38] suggests that these similar responses indicate the presence of a common triggering pathway that is antigen-independent, and that there are immunologic consequences occurring during irritant contact reactions.

89.2 PATHOPHYSIOLOGY OF INFLAMMATION

The pathophysiology of inflammation remains incompletely understood. Irritation is regulated by complex interactions between soluble mediators and cellular effectors, resulting in enhanced binding to adjacent surfaces, transmigration through matrices, release of cytoplasmic granules and vacuoles, activation or deactivation of genes, upregulation or downregulation of synthesis of specific cellular enzymes and products, and other changes in cellular metabolism.

Parenchymal cells of the local tissue (epithelium, fibroblasts), platelets, and vascular endothelium play a role in this complex mechanism of inflammation, but leukocytes (granulocytes, lymphocytes, and monocytes) are important. Other important components are soluble mediators that include free radicals, vasoactive amines, lipid mediators, cytokines, plasma-derived factors, and other molecules [39].

Recently research in cell signal transduction has made significant progress. Cell response to extracellular signals, such as hormones, antigens, cytokines, and growth factors, is mediated through a specific set of mechanisms (in ICD and ACD), the signal transduction cascade, that regulates cell function [40,41]. In this process different cellular components are recruited to stimulate and to regulate the inflammatory responses [16].

Two pathways are correlated to modulate irritant responses: direct effects of irritants on living keratinocytes and the damage to the skin barrier. Both activate biochemical signals that start the irritant reaction [42].

89.3 INVOLVED CELLS IN ACD AND ICD

Kanerva et al. [26] showed that the distribution of immunocompetent cells in allergic and irritant patch tests, using electron microscopy and monoclonal antibodies, indicates that these reactions cannot be differentiated. They also showed that in ACD and ICD, most inflammatory cells were OKT11 positive (pan T lymphocytes), and that the majority of these cells were also OKT4 positive (helper/inducer T lymphocytes) while the minority were OKT8 positive (suppressor/cytotoxic T lymphocytes). This concurs with the findings of Bruynzeel et al. [43].

Kondo and colleagues [44] evaluated the contribution of CD28 in contact hypersensitivity, and irritant reactions using CD28 gene-targeted mice demonstrated similar patterns of T-cell-derived cytokine in allergic and irritant dermatitis. They concluded that CD28 signaling affects the skin cytokine milieu and contributes to the full expression of both conditions, and that ACD and ICD share T-cell activation as a common pathway.

Allergic and irritant reactions induced by haptens and numerous compounds, respectively, can act in different pathways releasing cytokines at the beginning of contact reactions, causing early differences in the epidermal elemental content and expression of cytokines. Utilizing different compounds to induce irritation, a different cytokine expression was found by protein chain reaction (PCR): both substances used, nonanionic acid (NAA) and sodium lauryl sulfate (SLS), induced an increase in messenger ribonucleic acid (mRNA) for IL-1α, IL-1β, and IL-8; however, only NAA induced an increase in mRNA expression for IL-6. On the other hand, SLS, but not NAA, increased mRNA expression for granulocyte–macrophage colony-stimulating factor (GM-CSF) [45]. These findings indicate a time- and substance-dependent difference in the upregulation of mRNA for distinct cytokines in epidermis during the first 24 h of irritant reaction, and might be the effect of differences in the irritant's action on the cell membranes.

The differences seen in cytokine expression might be an effect of a different biological activity, differences in the characteristics of the primary agent that stimulate the initial response.

89.3.1 Cytokines, Interleukins, Lymphokines

Interleukins are secreted peptides and proteins that mediate local interactions between white blood cells (leukocytes) but do not bind antigen; those secreted by lymphocytes are also called lymphokines. Most interleukins have several sources, targets, and actions—this is especially the case for IL-1 and IL-6, which are also made of nonblood cells and act on many types of target cells other than blood cells; they are therefore more accurately called cytokines [46].

Lymphokines were previously restricted to the immune system, but recently it has been shown that these multitargeted molecules, such as eicosanoids, regulate cell growth and cellular differentiation mechanisms. Cytokines bind to specific receptors on target cells and thus regulate activation, proliferation, and differentiation of immune and nonimmune cells—complex network of interacting soluble products mediating immunologic and inflammatory reactions [47].

Cytokines are polypeptide mediators (low-molecular weight proteins transiently produced, which exert their biologic activities via specific cell-surface receptors) synthesized and released by cells at the site of inflammation to act on neighboring cells (paracrine action) or on the synthesizing cell itself (autocrine action), or into the blood to act on a distant site (hormonal action). The precise role of individual molecules during a specific inflammatory response is not yet determined. Most studies focus on the isolated activity of a particular cytokine. The function of various cytokines might change during the course of an inflammatory response, and the function of a specific cytokine must be interpreted in the context of many other factors that are simultaneously involved in this process, particularly because some of them appear to have paradoxic, bifunctional activities—stimulatory, inhibitory, or both [39].

Two major classes of cytokines are termed Th-1 and Th-2, on the basis of the predominant patterns produced by specific types of lymphocytes. In general, T lymphocytes designated Th-1 produce cytokines such as IL-2, TNF-γ, and gamma interferon (IFN-γ), whereas Th-2 type lymphocytes produce IL-4, IL-5, and IL-10. Th-1 cells promote cell-mediated immunity and inhibit the Th-22 cytokines, which conversely promote humoral immunity [38,48].

The primary cutaneous cytokines are members of the IL-1 and TNF family of molecules [47,49]. On the basis of *in vivo* modulation, studies by Issekutz et al. [50] and Barker et al. [51] propose that IL-2 and IFN-γ are the major factors in skin inflammation in both animal and human populations. Hoefakker et al. [11] demonstrated the involvement of Th-1 cytokines (IL-2 and IFN-γ) in the process of generation of both antigen-specific (allergic reaction) and non–antigen-specific (irritant reaction) T-cell responses. In the case of ICD, epidermal cells produce and secrete IL-1α and TNF-α. The cytokine release by the epidermal cells induces non–antigen-specific T-cell activation and subsequently IL-2, IFN-γ, and TNF- α production. Normally cytokines secreted by epidermal cells are not actively secreted by keratinocytes—many agents are capable of mediating keratinocyte cytokine production, including cytokines themselves [52].

Cytokines play a key role in inflammation, and functional polymorphisms in cytokine genes may affect responses to irritants; they mediate local interaction and distant communication among cellular elements of immune and inflammatory responses. As controllers, they act in cellular function and interaction via induced changes in the expression of adhesion molecules and receptor for cytokines [2]. Cytokines have been implicated in the pathogenesis of irritant dermatitis in animal models and *in vitro* [53]. Allen et al. [54] demonstrated that the TNF-α aminoacids (AA) is associated with susceptibility to experimentally induced ICD in response to sodium docecyl sulfate (SDS) and in low irritant-threshold individuals in response to benzalkonium chloride (BKC).

Differences in cytokine release and variation in individual sensitivity to cytokine stimulation may be possible factors that contribute to variations regarding irritant susceptibility in human populations [34].

Many cytokines increase during irritant reactions: TNF-α and IL-6 increased up to 10 times, and IL-1β, GM-CSF, and IL-2 increased up to three times. IL-1 has been reported to increase in ACD but not in ICD [31,55,56].

Various experimental techniques, for both human and rodent skin, show that specific types of cytokines are produced by epidermal cells after cutaneous barrier injury, and that there are immunologic consequences to the skin even after an induced irritant reaction [38].

89.3.1.1 Keratinocytes

Keratinocytes may participate in inflammatory and immune responses. These cells can be induced to express functional immunocompetent molecules, are capable of phagocytosis and motility in response to certain chemotactic factors, and secrete a wide range of inflammatory cytokines. Epidermal cells secrete cytokines with immunologic, inflammatory, and proliferative properties [52,57–59].

Keratinocytes are the principal source of cytokines in the epidermis and have been reported to secrete IL-1, IL-3, IL-6, IL-8, CSF, TNF-α, tumor growth factor (TGF)-α, TGF-β, and platelet-derived growth factor (PDGF); however, other epidermal cells such as LC, Th1+ cells, melanocytic cells, and Merkel cells are also capable of secreting cytokines, which include interleukins (IL-1, -3, -6, -8), colony-stimulating factors (GM-CSF, G-CSF, M-CSF), TGF-α, TGF-β, TNF-α, and PDGF [52].

Several cytokines, particularly IL-1α and TNF-α, bind to their receptors on keratinocytes and stimulate the production and secretion of additional cytokines, chemokines, and adhesion molecules, which modulate the function of other resident cell types, attract polymorphonuclear (PMN) leukocytes and T lymphocytes, and activate the infiltrating cells [60–68]. IL-1α, thought to be the most important biologically active cytokine in the IL-1 family, is probably preformed in the keratinocytes and released after cell membrane damage [69].

89.3.2 Tumor Necrosis Factor

TNF is stored in dermal mast cells [70], but following stimulation it may be produced by keratinocytes and LC [71]. An increasing evidence [72] suggests that TNF is functionally relevant to a variety of inflammatory skin diseases, in rodents and humans [73,74].

TNF-α and IL-1β are upregulated in both ACD and ICD and act directly to promote vascular permeability, leukocyte infiltration into an inflammatory site, and LC migration to the lymph node, and indirectly through upregulation of numerous other cytokines and chemokines and through changes in adhesion molecule expression, all critical steps in the development of inflammation and cutaneous hypersensitivity [37,75]. An important mechanism by which TNF influences the development of an inflammatory reaction is induction of the expression of cutaneous and endothelial adhesion molecules [74,76].

Corsini et al. [74] using skin irritants, such as SDS, or skin allergens, such as dinitrochlorobenzene and oxazolone, showed that all are capable of inducing TNF production.

89.3.3 Langerhans Cells

Fluctuations in epidermal LC density occur in such conditions as CD, other types of inflammatory skin reactions, and even after simple occlusion of the skin with water [3,27,29].

Even studies about histopathologic findings between ACD and ICD failed to demonstrate the differences. In an induced ACD and ICD study, Willis et al. [13] showed no differences on the characterization of the infiltrating cells and on the examination of LC in the early stages of inflammation.

Ferguson demonstrated, in a histometric study with lymphocyte subsets and LC in induced-patch reactions, a close similarity in the histologic appearance of allergic and irritant responses. This investigation confirmed previous reports [35,77–79].

Many aspects of the initiation and development of ICD and ACD remain poorly understood.

89.3.4 T Cells

The recruitment of leukocytes to sites of inflammation is a critical component of the response to tissue injury; thus, the elucidation of molecules mediating the extravasation of leukocytes from the periphery into the inflamed tissue is central to the understanding of the complex regulation of the host response to inflammation [80].

Two major classes of T cells have different functions. Cytotoxic T cells kill cells harboring harmful microbes, while helper T cells help activate the responses of other white blood cells, mainly by secreting a variety of local mediators, collectively called lymphokines, interleukins, or cytokines [46].

Recent studies focus on the subset of T lymphocytes participating in inflammatory skin reactions and also demonstrated a T helper (Th)2-like cytokine profile in nickel-mediated CD [81,82].

Ag presentation to the T cells is essential in the development of ACD, while irritant dermatitis is a form of toxic skin inflammation believed to be initiated by stimulating the release of proinflammatory mediators and cytokines, leading to the recruitment and activation of T cells [44].

As in ACD the predominant cell type involved in the mechanism of inflammation in ICD is the mononuclear cell, and in particular the T lymphocyte [13,21,25]. Initial recruitment of T lymphocyte immune surveillance machinery must be effected by cellular systems normally present in the skin, but which are nonspecific in that they cannot distinguish the specific (microbial) or chemical origin of the provoking material [18]. Brasch et al. [9] showed that the dermal infiltrate consisting of activated memory T cells and macrophages is nearly identical after 72 h when comparing allergic and irritant reactions.

89.3.5 Lymph and Skin Inflammation

A specific technique developed for collection of human skin-derived lymph promoted knowledge regarding immunologic processes involved in cutaneous-induced contact reactions. Authors suggest that signals detected in the lymph reflect immunologic reactions. This can be seen in a comparison of the IL-1β protein profiles in the course of ICD and ACD—their results demonstrated that it is not possible to discriminate between contact reactions in the allergic and irritant response types [83,84].

The large number of in part-activated and interacting immunocompetent cells migrating from the skin to the regional lymph nodes, as shown by Brand et al. [3,33], and the increased functional capacities of the lymph cells and complement proteins all point to an activation of the skin immune system in irritant skin processes.

Hunziker et al. [31] in a human study to investigate afferent skin lymph demonstrated significant increases of IL-6 and TNF-α in the early phase of an SLS-induced ICD. They demonstrated that in specific periods it is possible to find LC, monocytes, T and B lymphocytes, and granulocytes incubated in the lymph. These findings point to a possible role in the regulation of the immunologic response in the regional lymph nodes.

89.3.6 Neuropeptides

The neurologic system is capable of modulating various immunologic responses, including certain inflammatory events in the skin, by the secretion of neural-derived cytokines termed neurokinins [52,85–87]. There is increasing evidence that neuropeptides modulate various kinds of inflammatory reactions.

Gutwald et al. [88] demonstrated that neuropeptides increase plasma extravasation independent of the pathogenesis of inflammation. It is assumed that molecules such as substance P (SP), calcitonin gene-related peptide (CGRP), and somatostatin (SOM) are released from afferent neurons in the skin and participate in an immune response in the processes of ACD and ICD.

Taken together, irritant and allergic responses share many characteristics. The future of discriminating these resides in identifying unique aspects (a holy grail), a multifactorial quantitative approach, or both.

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90 Water Decontamination of Chemical Skin and Eye Splashes: Critical Review

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90.1 INTRODUCTION

Burns of all types result in significant morbidity and mortality. Skin/eye chemical burns are a significant problem. More than 25,000 chemical products including oxidizing agents, reducing agents, and corrosives have been identified as having the potential to cause burns (Liao and Rossignol, 2000). In 2001, the Bureau of Labor Statistics, US Department of Labor recorded 5,900 occupational deaths; 8.5% (502 deaths) of these were due to "exposure to harmful substances or environments," 68,269 nonfatal injuries were due to "exposure to harmful substances or environments," 25,125 nonfatal injuries involved "exposure to chemicals and chemical products," and 9,541 chemical burns were recorded (Bureau of Labor Statistics, 2004).

Josset et al. (1986) reported that there were approximately 7,000 serious occupational injuries annually from chemical burns in France, and about half of these involved the eyes. These burns resulted in 120,000 lost workdays and 250 cases of permanent disability.

In the United States, national data on exposures reported to poison centers are published by the American Association of Poison Control Centers in the Toxic Exposure Surveillance System. In 2002, there were 2,380,028 total human poison exposure cases reported, with 1,153 deaths. There were 193,822 dermal exposures and 130,857 eye exposures (Watson et al., 2003).

Following the removal of contaminated clothing which has been said to decrease chemical skin decontamination by up to 80% (McMullen and Jones, 1998), standard references recommend water or normal saline for immediate decontamination of skin/eye chemical splashes with the addition of soap if the chemical substance is lipid soluble (McMullen and Jones, 1998; Standing and Caniano, 1996; NIOSH, 1997; Coe and Douglas, 1994). Soap should not be used in the eyes. Older literature suggests that immediate flushing of the eyes for about 30 min from the nearest shower or faucet should be done following sodium or ammonium hydroxide ocular exposure (Stanley, 1965). In the United States, the Occupational Safety and Health Administration (OSHA) regulations mandate emergency eyewash stations and quick-drench showers in all facilities where potentially dangerous chemical agents are present (Liao and Rossignol, 2000). Most of these emergency facilities utilize water for skin/eye decontamination.

Lewis (1959) recommended initial copious water decontamination, followed by neutralization (1/2 ounce of sodium

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bicarbonate dissolved in 1 pint of water for acids; 1% sodium citrate for alkalis), followed by a second decontamination with copious amounts of water for skin splashes.

90.2 MATERIALS AND METHODS

In-depth electronic and paper literature searches were performed to retrieve pertinent articles involving water decontamination of skin/eye chemical splashes and chemical burns. Searches were done in the National Library of Medicine MEDLINE and TOXLINE databases using combinations of search terms such as "chemical burns," "skin burns," "dermal burns," "eye burns," "ocular burns," "occupational burns," "workplace burns," "chemical decontamination," "skin decontamination," "eye decontamination," and "ocular decontamination." For older literature, hardcopy versions of Index Medicus were reviewed back to 1929. The references/bibliography section of each retrieved paper was also reviewed for any pertinent references.

Organization web pages, such as that of the American Burn Association (American Burn Association, 2003), were reviewed for data on the occurrence and etiology of chemical burns, and for references/links to other sources of chemical burn data such as State Health or Health and Environment departments. These web pages were also reviewed. Chemical burn occurrence and outcome data from the U.S. Department of Labor Bureau of Labor Statistics website were also reviewed. Internet Google searches were also conducted using all combinations of the above search terms.

90.3 RESULTS

One estimate stated approximately 5,511 deaths associated with fire and burns in the US in 1991 (Brigham and McLoughlin, 1996). Of these, 125 deaths were said to be due to hot liquids, substances, and objects (including caustics and corrosives) and contact with these accounted for nearly 500,000 emergency department visits (Brigham and McLoughlin, 1996; Linares and Linares, 1990; Monalfo, 1996). In a 1977 University of Michigan Quality of Employment Survey of 36 illnesses and injuries and 17 job hazards in 1,515 workers, chemical burns were in the top three illness and injury complaint categories among employed men (Leigh, 1989). While chemical burns account for only about 3% of all burn injuries, they are responsible for over 30% of burn deaths (Demir et al., 2003).

90.3.1 Occupational Burn Information Collected by Governmental Agencies or Assembled from Government Sources

West Anglia and Oxford Region, UK: In four UK counties in the West of Anglia and Oxford region, Wilkinson (1998) reviewed the epidemiology of burn patients treated in accident and emergency departments or admitted to the hospital for burn care during 1994–1995. About half of the burn patients admitted to the hospital were treated in burn units, one-fourth were treated on plastic surgery wards, and the remainder were admitted to specialty units including trauma, orthopedics, pediatrics, and ophthalmology. The largest numbers of admitted patients were in the working age group, which was also the largest group in the general population. Burn patients accounted for approximately 1% of cases seen in accident and emergency departments and about 10% of these patients were admitted to the hospital, with a mean length of stay of 7.5 days (Wilkinson, 1998).

South Wales, UK: Munnoch et al. (2000) studied work-related burns in South Wales during a 2-year period between 1995 and 1996. There were 324 cases of work-related burns, and records were available for 319 of these. Twenty percent of all burns referred to the burn center in Swansea occurred in the workplace. Chemical burns were the most frequent cause (23%) with caustic soda exposure in 21 cases, cement in 15 cases, and various acids and alkalis accounting for the remaining 37 cases. Of these 319 patients, 175 were admitted to the burn center and 79 required surgery. The mean length of hospital admission was 8.5 days (range 1–110 days), representing overall 1,485 hospital days. Males aged 16–40 years comprised 70% of the patients with work-related burns. Fifty-five percent of patients with work-related burns were admitted to hospital and approximately 25% required surgery (Munnoch et al., 2000).

Switzerland: de Roche et al. (1994) studied the epidemiology and costs of work-related burn injuries in Switzerland. They noted that about 4.6% of all accidents in Switzerland are burns and that 3.0% of all accidents are work-related burn injuries. Estimates based on population suggest that there are 36,000 burns annually in Switzerland with 5% requiring hospital admission and one-third of these being treated in a burn center. There is a compulsory insurance program for workers in Switzerland that covers accidents both on and off the job. In 1984, 6,814 burn accidents were covered by this insurance program, 58% work-related and 42% non-work-related. The total cost for the burns was 17.7 million Swiss Francs, with 19% for medical care, 34% for salaries while off work, and 46% for annuities (de Roche et al., 1994). The proportion of chemical burns was not reported.

Taiwan: Chien et al. (2003) studied the epidemiology of hospitalized patients with burns in Taiwan during a 2-year period from 1997 to 1999: a total of 4,741 patients were hospitalized for burn treatment. Work-related burns occurred in 1,459 patients (30.8%). Among adult patients, chemical burns due to exposure to corrosive agents such as strong acids or alkalis accounted for 9% of the injuries. Burns due to explosions and chemical contact occurred more frequently in the workplace (32.9%), were more serious (average 25% total body surface area [TBSA]), and resulted in longer average hospital admission times (23 days) (Chien et al., 2003).

US NIOSH/CPSC: Over 3 months in 1981, the US National Institute for Occupational Safety and Health (NIOSH) and the Consumer Product Safety Commission (CPSC) conducted a surveillance program of occupational injuries treated at a sample of 66 U.S. hospital emergency departments (MMWR, 1981). There were a total of 2,747 burn injuries (cause not

specified) that represented 4.5% of the total 61,585 occupational injuries treated in these facilities. The most common sites of burns were face, arm, and trunk. Based on the surveillance system, it was estimated that there were 3.3 million occupational injuries treated in the US emergency departments in 1981 (MMWR, 1981). If the percentage of burns were constant, this would represent 148,500 occupational burn injuries yearly. Chemical burns were not listed separately from all occupational burns.

New England, USA: Rossignol et al. (1989) collected data on burned patients aged 20 years or older admitted to any of 240 of New England's 256 acute-care hospitals for treatment of a new burn injury. Chemical burns were among the type of injuries included in the study. Overall, 1,614 new burn injuries were identified during the 1-year study period between 1978 and 1979. Of these, 485 burns (30%) were work-related. Overall, 40% of the 1,133 burns in males were work-related while only 7% of the 481 burns in women were work-related. There were 91 chemical burns in males, of which 67 (74%) were work-related.

State of North Carolina, USA: Hunt et al. (2000) in a survey of occupation-related burn injuries during 1994 using data from the US National Census of Fatal Occupational Injuries (fatal cases) and the North Carolina Department of Labor (nonfatal cases) found that there were 34 burn deaths (15.3%) and 1,720 nonfatal burns. Burn injury was the fourth most common cause of workplace deaths, but what proportion of these were chemical burns was not specified. Of the nonfatal burns, 709 (41.2%) were caused by chemical exposure. Involved chemicals were alkalis (20%), cleaners and solvents (16.9%), propane (12.2%), halogens (7.0%), inorganic and other acids (3.6%), hydrocarbons (2.0%), and other chemicals (38.3%). Chemicals and chemical products were the most common agents causing workplace burn injuries (Hunt et al., 2000).

State of Washington, USA: In a study of occupational burns in Washington State during a 5-year period of 1989–1993, there were 27,323 workers' compensation claims for work-related burns; 26.8% (7,323) of these were chemical burns (McCullough et al., 1998). These authors note that in 1994, the U.S. Bureau of Labor Statistics reported that there were 53,800 occupation-related burns resulting in lost work time and that studies of work-related burn patients admitted to burn centers ranged from 21 to 30% of all admissions and accounted for 20–30% of all serious burns (Mccullough et al., 1998). Of exposures, 2,173 (8%) were to unspecified chemicals, 906 (3.3%) to soaps and detergents, 604 (2.2%) to solvents/degreasers, 462 (1.7%) to calcium hydroxide, 451 (1.6%) to sulfuric acid, 488 (1.6%) to chlorine compounds, and 371 (1.4%) to sodium hydroxide. Industries at the highest risk for chemical burns were hazardous waste landfill clean-up; portable cleaning and washing; pulp and paper manufacturing; and chemical blending, mixing, and manufacturing (Mccullough et al., 1998).

State of Washington, USA: Baggs et al. (2002) investigated work-related burns in Washington State during 1994–1998. There were a total of 20,213 work-related burn claims accepted by the workers' compensation system during this period, but only 1.5% of burned workers were admitted to the hospital. However, this 1.5% of burned workers represented 55% of the cost incurred. The costs for all work-related burns were in excess of US$5 million annually. Patients hospitalized for work-related burns lost an average of 132 workdays, while burned workers not requiring hospitalization lost an average of 3 workdays. They noted that the workers' compensation data underestimated the frequency and cost of work-related burns (Baggs et al., 2002). Burns were evaluated in two categories: thermal and chemical. Among previously identified high-risk industries were hazardous waste clean-up and the chemical industry. In this study, chemical mixing and manufacturing, concrete work, and construction ranked high as industries having hospitalized work-related burn cases; janitorial services was also an industry having chemical burn cases reported which had not been identified in previous studies (Baggs et al., 2002).

State of Utah, USA: The Utah Department of Health, Bureau of Epidemiology collected data on work-related burns during 1997 (Utah Department of Health, 1997). In 1997, there were 699 hospital admissions for burn treatment, of which 133 were work-related. Males accounted for 82% of these cases. Workers aged 25–44 had 60% of all work-related burns (Utah Department of Health, 1997).

State of Colorado, USA: The Colorado Department of Health and Environment noted that over a period of 1980–1998 an average of 24 state residents died each year from burn injuries (Colorado Department of Public Health and Environment, 2003). Approximately 330 Colorado citizens are hospitalized yearly for burn injuries, and approximately half of these are due to scalds, hot objects, or exposure to caustic substances (Colorado Department of Public Health and Environment, 2003).

State of Massachusetts, USA: Rossignol et al. (1986) studied the epidemiology of work-related burn injuries in Massachusetts that necessitated hospital admission during a 1-year period in 1978–1979. Of 825 total burn admissions, 240 (29%) were work-related. Of the work-related burns, 95% were in males. There were 29 chemical burns that accounted for a total of 248 hospital admission days.

State of Ohio, USA: Chatterjee et al. (1986) studied 199 burn injuries in northeastern Ohio evaluated in an emergency department during 1977, representing 2.4% of all patients evaluated for any type of trauma. The cause of the burn was known in 187 cases (94%). Of these, 124 (66%) were due to hot substances, corrosive liquids, or steam (not further delineated). Of these patients, 55 had a work-related burn and 52 claimed eligibility for workers' compensation (Chatterjee et al., 1986).

90.3.2 Burn Center/Unit Data without Information on Decontamination

France: A review of survival rates in patients hospitalized in French burn centers during 1985 was performed by Wassermann and Schlotterer (1989). A total of 2,398 patients were admitted for treatment to 17 French burn centers and

there were 238 deaths for an overall mortality rate of 11.8%. Young adults constituted the majority of admissions (greater than 50%) and the authors noted that withdrawal of these persons from economic activity for weeks or months was significant (Wassermann and Schlotterer, 1989).

Hobart, Tasmania, Australia: Ricketts and Kimble (2003) reviewed 31 patients with chemical burns admitted to a burn center in Hobart, Tasmania between 1989 and 1999. Of these, 38% occurred in an industrial setting and the most common chemicals involved were cement (25%), sulfuric acid (16%), and hydrofluoric acid (16%). The mean TBSA affected was 3.5% and the mean hospital admission time was nine days (range 1–30 days).

Brisbane, Queensland, Australia: Pegg et al. (1986) reported a series of occupation-related burns admitted to a hospital in Brisbane, Queensland over a 7-year period between 1976 and 1983. There were 182 occupation-related burn patients of whom 95% were males. There was one fatality. Half of these burns occurred in males less than 30 years old. Ocular burns were present in 5.5% of these patients and were caused by chemical exposure, gas explosions, and electrical flashes. Eighteen patients in this series had chemical burns that were less than 20% (mean 2.25%) of the TBSA. Sulfuric acid and caustic soda were the most common causes of chemical burns. Perchloroethylene, ammonia, cresylic acid, hydrofluoric acid, and Shellite (white gas; Coleman Fuel; a refined petroleum product) also caused chemical burns. The eyes and hands were the main burn sites (Pegg et al., 1986).

Copenhagen, Denmark: In a study of occupational burn injuries treated in the municipality of Copenhagen, Denmark during a 1-year period from 1982 to 1983, 371 patients had work-related burn injuries (Lyngdorf, 1987). Of the 361 patients treated in casualty wards, 70 were subsequently treated in the burns unit, six as inpatients and the rest as outpatients. There were 24 patients with corrosive chemical burns, 18 of which (75%) occurred in the chemical industry. The involved chemicals were alkalis (13), acids (7), and other chemicals (4) (Lyngdorf, 1987).

Dublin, Ireland: Carroll et al. (1995) reviewed the records of 100/120 patients admitted to a burn center in Dublin, Ireland during a 3-year period from 1988 to 1991. Of these 100 patients, 2 had corrosive burns.

Toronto, Ontario, Canada: Ng et al. (1991) performed a 6-year retrospective study of 193 work-related burns in patients treated in a burn center in Toronto, Ontario between 1984 and 1990. Of these, 94.3% were males and 64.2% were under the age of 35 years. Chemical burns accounted for 5.1% (10 cases) of the total. Chemicals involved were alkalis (sodium hydroxide, "detoxification agent," pulp decomposing agent, unknown) (6 cases) and acids (hydrofluoric acid, sulfuric acid, phenol) (4 cases). The mean TBSA chemical burn was 6.0% (range 1–98%). The total cost of hospital treatment for these 193 work-related burns was Can$2.96 million and the estimated time lost from work were 439 workdays.

Tehran, Iran: Lari et al. (2000) reviewed 3,341 burn patients admitted to a burn center in Tehran, Iran during a 3-year period between 1995 and 1998. Of a total of 110,554 patients evaluated, 3,341 (3%) were admitted to the burn center. Of admitted patients, 124 (3.7%) incurred the burn in the workplace. A total of 67 burns (2%) were due to chemical exposure.

Madras, India: In a study of 1,368 patients evaluated for burns in a Medical Hospital in Madras, India during a 1-year period from 1987 to 1988, there were 135 work-related burns (Jayaraman et al., 1993). Of these 135 work-related cases, 18 were due to chemical exposures.

Digboi, Assam, India: Sarma and Sarma (1994) performed a retrospective study of 348 burn patients admitted to a peripheral industrial hospital in Digboi, Assam, India during a 10-year period between 1980 and 1990. Overall, work-related burns comprised 12% of the study group. There were 20 chemical burns (5.7%) from exposure to acids and alkalis. Of 42 work-related burns, five were due to acid exposure (Sarma and Sarma, 1994).

San Diego, California, USA: In a study of 232 cases of all types of occupational burns treated in a burn center in San Diego from 1977 to 1982, chemical burns accounted for 4% of patients (Inancsi and Guidotti, 1987). Of admitted chemical burn patients, 50% were permanently disabled, the median hospital stay was 12 days, and the median time from hospital discharge to return to work was 13 days in those who were not permanently disabled. While chemical burns were not common, they were often severe (Inancsi and Guidotti, 1987).

Atlanta, Georgia, USA: In a study of 844 burn Center admissions in Atlanta over approximately 3.5 years from 1987 to 1990, there were 33 chemical burns (3.9%) with a mean TBSA burn of 9.0% and an overall survival of 90.9% (Renz and Sherman, 1992). There were three deaths from chemical burns.

90.3.3 Burn Center/Unit Data with Information on Decontamination and Clinical Outcome

Toronto, Ontario, Canada: In a review of chemical burn patients admitted to a regional burn center in Toronto, Ontario over an 8-year period, the 24 chemical burn patients comprised 2.6% of all admissions (Cartotto et al., 1996). Work-related accidents accounted for 75% of these burns, with the involved chemicals being hydrofluoric acid, sulfuric acid, black liquor (a heated mixture of sodium carbonate, sodium hydroxide, sodium sulfide, sodium thiosulfate, and sodium sulfate), various lyes, potassium permanganate, and phenol. Of these 24 patients, 14 required extensive excision and skin grafts. Complications were frequent (58% of patients), including eye contact with the chemical, wound infections, tendon exposures, toe amputation, and systemic toxicity from chemical absorption. One patient with a chemical scald burn involving 98% of the TBSA died. In 14 of 24 patients (58%), removal of contaminated clothing followed by immediate water shower decontamination was done; five other exposed patients did not have these interventions. Five of the eight eye splash cases had immediate decontamination

at the site (presumably with water). While the three eye splash patients who did not have immediate decontamination developed prolonged conjunctivitis, three of the five who were decontaminated immediately developed corneal erosions and one who had eye exposure to black liquor had a very deep corneal erosion, which resulted in blindness (Cartotto et al., 1996). Despite immediate decontamination of the skin or eyes with water, some of these patients developed burns and significant complications.

Chandigarh, India: In a study of 27 cases of acid and alkali burns evaluated over a 5-year period, Sawheny and Kaushish (1989) noted that chemical injuries differed from thermal injuries. Of 562 patients admitted to a burn center over a 5-year period, 16 were acid exposures (sulfuric or nitric acid) and 11 were caustic soda (sodium hydroxide) exposures. The 11 patients exposed to caustic soda were involved in the collision of a tank truck with a passenger bus. The majority of chemical burns (20/27; 74%) involved less than 15% of the TBSA and 81.5% were full thickness burns, mainly on the face, upper trunk, arms, and hands. Eye involvement was present in 74% of patients and both eyes were involved in 15%. Severe conjunctivitis was present in all patients with eye exposure, with keratitis and corneal ulcerations progressing to opacities occurred in 63% of patients. Corneal perforation progressing to panophthalmitis and vision loss occurred in two cases. Severe eyelid ectropion developed in 12 patients (44%). By the end of the third week, wound infections developed in two-third of patients and all wounds were infected by 4 weeks after surgery. Invasive sepsis occurred in only one patient. In those patients, "… thorough and continuous irrigation of the area of damaged tissues with copious volumes of water …" was done as early as possible, although this may have been after a delay of hours following exposure, but was noted to be of limited effectiveness when the patients did not arrive until after a delay of days (Sawheny and Kaushish, 1989).

Boston, Massachusetts, USA: In a study of 857 inpatients treated at a burn center in Boston during a 4-year period from 1976 to 1980, 35 (4%) had chemical burns (Leonard et al., 1982). The chemicals involved were acids (sulfuric, hydrochloric, hydrofluoric, carbolic, chlorosulfonic, trichloroacetic) (15 cases), alkalis (lye, cement) (9 cases), and other/unknown substances (10 cases). The mean TBSA burn was 8.7%, the mortality was 6% (two patients), and the length of hospital admission was 15 days. The injury occurred in the workplace in 18 cases (51%), in the home in 10 cases (29%), and was due to a deliberate chemical assault in seven cases (20%). Sixteen patients had immediate water decontamination (within 10 min of exposure and lasting at least 15 min) and 19 had delayed water decontamination. The delayed contamination group had a fivefold greater incidence of full-thickness burns and a significantly longer duration of hospital admission, despite the fact that the mean TBSA burn in the delayed decontamination group was one-half that of the immediate decontamination group (Leonard et al., 1982). Although immediate water decontamination appeared to decrease burn severity, it was unable to completely prevent them and the 16 patients in this category still required hospital admission for a mean of 7.7 days and 12.5% of them had full-thickness burns.

Iowa City, Iowa, USA: In a review of patients with chemical burns admitted to a burn Center, of a total of 2,762 patients 94 (3.3%) had chemical burns (Wibbenmeyer et al., 1999). Of the chemical burn patients, 31 (34%) were due to anhydrous ammonia. Chemicals involved included acids (14 cases), bases (68 cases), inorganic agents (2 cases), organic agents (6 cases), and unknown (5 cases). The majority had work-related burn injuries. Patients either had immediate water decontamination at the incident site or underwent water flushing in the burn center until the skin pH returned to normal. Further water irrigation was done if discomfort recurred and daily hydrotherapy was performed. There was one fatality in this group, and 36/94 (38%) required skin grafting with five patients having multiple procedures. Complications including wound infections, pneumonia, cardiac failure, cardiac arrhythmias, and myocardial infarction occurred in 24/94 (25.5%), and sequelae were noted in 27/94 (28.7%) (Wibbenmeyer et al., 1999). Early and prolonged water decontamination did not prevent serious burns from developing.

90.3.4 Experimental Animal Studies

Older experimental animal literature supports immediate and even prolonged (up to 8 h) continuous irrigation with water for acid or alkali chemical burns, noting that the sooner it is begun, the more likely it is to be effective (Davidson, 1927; Gruber et al., 1975; van Rensberg, 1962). Delays to initiation of water decontamination of as little as 5–30 s sometimes make significant differences in its ability to prevent or decrease the severity of burns (Gruber et al., 1975; van Rensberg, 1962). Of note, statistical comparisons of various treatment modalities are generally lacking in these older studies.

One of the earliest series of experimental animals studies was by Davidson (1927) who investigated acid (50 and 70–71% nitric acid; 10, 25, 50, and 96% sulfuric acid; 37% hydrochloric acid; 99% acetic acid; saturated and half-saturated trichloroacetic acid, and alkali; 50% sodium hydroxide; 50% potassium hydroxide) burns in rats by immersing a hind leg in the solutions for various periods from 15 s to 1 min. At a higher concentration of acids and bases, many animals receiving no decontamination died and developed severe burns. Neutralization with 5% sodium bicarbonate (acid burns) or 1% acetic acid (alkali burns) was compared to water decontamination either by holding the exposed limb under running tap water or by placing the rat in a large tank of water. In all cases, animals treated with neutralization developed less severe burns than untreated controls, while animals decontaminated with water developed less severe burns than those treated with neutralization (Davidson, 1927). However, all animals except those exposed to 10% (no burns even in untreated controls) and 25% (no burns in animals receiving either neutralization or water decontamination) sulfuric acid developed burns, regardless of treatment modality.

Rats with skin exposure to 2 N sodium hydroxide were washed with 500 mL of distilled water at a rate of 33 mL/min beginning at 1, 10, and 30 min after exposure (Yano et al., 1993). Subcutaneous tissue pH was measured at 1 min intervals for up to 90 min following exposure. The peak tissue pH measurements were 7.97 for the 1 min group, 10.57 for the 10 min group, and 12.17 for the 30 min group. The pH measurements for the groups with water washing begun at 10 and 30 min postexposure were not different from those seen in exposed controls that received no decontamination (Yano et al., 1993). Using the same rat model, a comparison was made of subcutaneous tissue pH between rats washed with 500 mL of distilled water and 500 mL of a 0.35 M sodium citrate solution as a neutralizing agent (Yano et al., 1994). As compared with the water decontamination group, subcutaneous tissue pH measurements were significantly higher in the 1 min group and significantly lower in the 10 and 30 min washing groups. Regardless of which decontamination measure was used, the upper layers of the skin became necrotic and deeper burns were observed in the groups with delayed (10 or 30 min) beginning of decontamination (Yano et al., 1994).

Brown et al. (1975) while studying the efficacy of polyethylene glycol (PEG) for decontamination of phenol and related compounds exposure in rats, also compared PEG with water for decontamination of 45% sodium hydroxide concentrated sulfuric acid exposures. Water decontamination was more efficacious than PEG, although burns of varying severity developed in all animals exposed to sulfuric acid and decontaminated with water, while burns of varying severity developed in 12/90 animals exposed to 45% sodium hydroxide and decontaminated with water (Brown et al., 1975).

Andrews et al. (2003) challenged the dogma that skin burns caused by alkaline substances should be decontaminated with water and that neutralization should not be attempted. In an experimental animal study of rats exposed to 2 N sodium hydroxide, decontamination with 5% acetic acid was superior to water decontamination at attaining a physiological pH, resulted in less severe tissue damage, and was associated with improved wound healing, thus supporting the idea that perhaps water decontamination is not the best initial intervention in alkaline chemical burns (Andrews et al., 2003).

90.3.5 Older Human Case Reports

Older literature also describes attempts at chemical neutralization following skin/eye chemical splash exposures. Terry (1943) described the use of a 5% ammonium chloride solution for neutralization with sodium bicarbonate followed by plain water washing which did not prevent significant burns. While no actual patient data were presented, this author stated that immediate 5% ammonium chloride solution irrigation "… in the great majority of cases prevented a burn; however, skin burns did occur when there was more than a 30–40 s delay before ammonium chloride irrigation was begun. For caustic soda eye splashes, this author recommended a 5 min irrigation with 5% ammonium chloride solution followed by a 55 min irrigation with boric acid/saline solution, which "… has reduced the time of recovery of caustic soda burns of the eye from weeks to days" (Terry, 1943).

Cason (1959) described three cases of extensive industrial burns. The first patient fell into a vat of a cresylic acid derivative and developed burns over 15% of the TBSA. Despite immediate removal of contaminated clothing and thorough water shower decontamination, he developed anuria, hypokalemia, and cardiac failure and died on the 10th day after the accident. A second patient slipped and inadvertently immersed his arm in a vat of chromic acid, developing an extensive burn of the entire limb despite immediate water washing. About 45 min later, the arm was again decontaminated with a phosphate buffer solution. Extensive excision and delayed skin grafting was required, requiring 44 days in hospital. A third patient fell into a vat of hot (81°C) nickel-plating solution (nickel chloride, nickel sulfate, boric acid, cumarin). Sodium bicarbonate was applied at the company infirmary and 40 min later a "buffer solution" compress was applied. Burns involved 40% of the TBSA. Hypotension, oliguria, and hyponatremia developed and the patient expired on the fifth day following the accident.

90.3.6 More Recent Human Case Reports

With relatively dilute sodium hydroxide (4%) oven cleaner aerosol exposure, full thickness burns of the face requiring skin grafting may occur despite the lack of early pain (Lorette and Wilkinson, 1988). A patient with such an exposure did not present to the emergency department until 2 h after aerosol exposure. The patient had wiped her face with a water-moistened cloth immediately after exposure, but did not irrigate her face with water. Continuous water irrigation in the emergency department did not significantly modify the pH of the patient's facial skin nor prevent development of full-thickness burns requiring skin grafting (Lorette and Wilkinson, 1988).

A 20-year-old man fell into a caustic lime pit and developed an 85% TBSA burn (Erdmann et al., 1996). Treatment was delayed by more than 20 h because of initial misdiagnosis and confusion over what the exposure actually involved, and he arrived at the burn center still covered with a thick, adherent layer of the lime. Decontamination in a water-filled Hubbard tank was only partially successful. Skin grafting was completed by 30 days after the injury, although some areas had to be secondarily debrided and regrafted with permanent wound closure obtained only after 2 months. Functional restoration was achieved at 3 months after injury (Erdmann et al., 1996).

O'Donoghue et al. (1996) described three cases of caustic soda burns of the hands or feet. In two cases, the patients had immediate copious water decontamination; in the third case decontamination was not described. All three patients developed significant, deep neurotic burns requiring debridement and skin grafting. In two cases, recurrent necrosis occurred over 6 and 13 months.

A 36-year-old male was exposed to sodium hydroxide (pH 12–14) from a spilled barrel in a leather-processing factory (Acikel et al., 2002). Contaminated clothing was immediately removed and the worker was washed with water continuously in a shower for approximately 20 min. On admission to the burn center, there were 53% TBSA burns present. Irrigation with water was done for a further 2 h, and repeated six times with 4 h rest periods in between. Debridement and skin grafting were required over a 43-day hospitalization, following which pressure garments were worn for 18 months. No functional deficits were noted at the conclusion of treatment.

Paulsen et al. (1998) described two workers sprayed with liquid titanium tetrachloride while dismantling piping in a chemical plant. Titanium tetrachloride reacts with water releasing heat and hydrochloride acid, and should initially be dry-wiped from exposed areas before other decontamination is attempted. These two workers were perspiring heavily and the titanium tetrachloride reacted with the perspiration, releasing hydrochloric acid. They were immediately dry-wiped with towels and then decontaminated with water in safety showers. On hospital admission, they had 18 and 20% TBSA partial thickness burns. One also had bilateral corneal burns. Both required debridement and skin grafting, and remained in the hospital for 2 weeks. Return to light duty was allowed after 8 weeks (Paulsen et al., 1998).

Seven Saudi Arabian children had skin exposure to sulfuric acid when they tipped over a drum stored on the rooftop of their residential block (Husain et al., 1989). These children developed chemical burns of 3–60% TBSA. Contaminated clothing was not removed and water decontamination was not done until one-half hour after exposure. Following this, four children were treated and released and three children with 10, 15, and 60% TBSA burns were hospitalized. The child with 60% TBSA burns had 166 days of initial hospital admission, eight autografting and one homografting procedures, and two further hospital admissions and surgical procedures for burn sequelae. In these seven children, one-half hour of water irrigation begun one-half hour after sulfuric acid skin exposure did not prevent burns and significant sequelae in one child.

Two workers had similar skin and inhalation exposures to liquid anhydrous ammonia and its vapor when a hose became disconnected during transfer from a river barge to a dock tank (Latenser and Lucktong, 2000). One worker immediately left the area, showered with water, and removed contaminated clothing. He developed bilateral corneal burns, edematous and peeling lips, and hyperemia of the face and neck. After a 1-day hospital admission, rapid healing occurred. The second worker did not change contaminated clothing or shower immediately. On hospital arrival 90 min later, the face and lips were swollen, breathing was difficult, the pharynx and vocal cords were swollen, and endotracheal intubation was required to maintain a patent airway. There were 14% TBSA partial thickness burns on the face, neck, chest, arms, hands, and thigh. Skin grafting was required and wound infection developed in the thigh burn. Hospital admission was for 13 days. Although immediate water decontamination was associated with less severe burns in one of these two workers, it did not prevent burns from developing.

90.3.7 Case Series/Epidemiological Studies

In a study of 51 patients, to evaluate whether immediate water decontamination was adequate, patients were divided into groups and adequate treatment was defined as immediate dilution or neutralization therapy (Sykes et al., 1986). The *mortality* in this study was 13%, most of the chemical burn patients were young men, and the injury was most often work-related. The largest group of chemicals involved were alkalis (sodium and potassium hydroxides) followed by sulfuric acid, gasoline, anhydrous ammonia, white phosphorus, and hydrofluoric acid; four of the five *deaths* were work-related. Five of 38 deaths were caused by chemical burns and four of 23 (17%) were work-related. Although the group with immediate water decontamination had a generally shorter length of burn center admission and decreased mortality, this intervention did not prevent the development of burns or a 9.5% mortality (Sykes et al., 1986).

Bromberg et al. (1965) reviewed 273 chemical burns treated at two hospitals in New York during 1957–1963. Accidental exposures and deliberate assaults with caustic substances were approximately equally represented. Alkalis (potassium hydroxide, sodium hydroxide) were involved in 208/273 cases (76%) and resulted in more severe burns than did acids. The head and neck were most often involved and a high percentage of patients had concomitant corneal burns. A subset of 85 patients were further described, either receiving continuous water irrigation in a shower, brief water irrigation (30–60 min), continuous water soaks, or open treatment. Continuous water irrigation in a shower decreased the waiting period until skin grafting could be done to 22 days as compared with 26–34 days with the other treatment modalities. It also decreased the average length of hospital treatment to 19 days as opposed to 23–39 days with the other modalities. The requirement for skin grafting was 20% less in the group receiving continuous water irrigation (Bromberg et al., 1965). Hydrotherapy, especially by immersion, has been associated with sepsis and alternative local wound care with sterile solution and patient isolation maintenance has been shown to decrease lethal infectious complications (Shankowsky et al., 1994).

Wolfort et al. (1970) reported a series of 416 patients with lye injuries treated at two hospitals in Baltimore from1952 to 1968. Of these, 42 had cutaneous lye burns involving from 5 to 60% of the TBSA. Only nine injuries resulted from workplace accidents; the majority were deliberate assaults. The mean hospital admission time was 32 days and one fatality occurred but was attributed to an anesthetic accident rather than the burn itself. Complications included tympanic membrane perforations (from lye running into the external auditory canal), parotid fistulas, a greater potential for keloid formation than seen with thermal burns, and the early appearance of Marjolin's malignant ulcers in the burn scars (seen at 3–9 years following lye burns and an average of

34 years following thermal burns). Treatment protocols at these two hospitals included early water decontamination followed by 12–24 h of continuous water irrigation in a shower (Wolfort et al., 1970). Despite these interventions, all 42 patients developed burns requiring debridement and skin grafting.

Curreri et al. (1970) described 111 patients with chemical burns treated in the U.S. Army Institute of Surgical research over a 19-year period from 1950 through 1968. Of these, 96 patients had white phosphorus burns, 5 were burned with concentrated sulfuric acid, 3 were burned with lye, 3 with mustard gas, and 4 with other chemicals. There was a longer healing period for patients with chemical burns than for those with burns from other etiologies, although the mortality was less: 5.4% for patients with chemical burns as compared to an overall burn patient mortality of 10.5%. There was a high incidence of periorbital and ocular complications in the chemically burned patients.

Mozingo et al. (1988) described 87 patients with chemical burns treated at the U.S. Army Institute of Surgical Research during a 17-year period between 1969 and 1985. The most common chemical involved was white phosphorus (49 cases). Other causative chemicals were acids (13 patients), alkalis (10 patients), organic solvents (5 patients), and a variety of other chemicals (15 patients). Patients with chemical burns from other than white phosphorus had shorter hospital admission times than other burn patients. In the 38 patients with other than white phosphorus chemical burns, the average TBSA was 29.8%, the average third-degree TBSA burn was 17.8%, and there was a 26.3% mortality. Three of these patients had associated eye injuries. Complications were noted in the nonwhite phosphorus chemical burn patients, including joint contractures (3 cases), cellulites (6 cases), septicemia (3 cases), upper GI bleeding (1 case), pneumonia (2 cases), burn wound infection (2 cases), blindness (2 cases), myocardial infarction (2 cases), phenol toxicity (2 cases), pulmonary embolus (2 cases), brain death (1 case), formate toxicity (1 case), nitrate toxicity (1 case), pancreatitis (1 case), and other (7 cases).

In patients with burn injuries treated at the U.S. Army Institute of Surgical Research from 1963 to 1968, 104 cases with ocular burn injury were identified (Asch et al., 1971). Flame injury was the most common etiology, but chemical exposure was the cause in 27 patients.

90.3.8 Hydrofluoric Acid Burns

Hydrofluoric acid (HF) is a relatively weak acid that penetrates deeply into the tissues resulting in severe burns; high concentrations can also cause life-threatening systemic poisoning and fatalities (Dayal et al., 1994; Beaudouin et al., 1989; Camarasa, 1983; Muriale et al., 1996; Tepperman, 1980; Sheridan et al., 1995; Kirkpatrick et al., 1995; Mullett et al., 1987; Chan et al., 1987). Penetration of H^+ and F^- ions into the tissues causes the corrosive lesions; chelation of calcium leads to systemic hypocalcemia (Noonan et al., 1994; McCulley et al., 1983) and other serious electrolyte imbalances (hypomagnesemia, hyperkalemia) may occur (Upfal and Doyle, 1990; el Saadi et al., 1989) leading to severe metabolic acidosis, cardiovascular shock, cardiac conduction abnormalities, and cardiac arrhythmias including nonperfusing Torsades de Pointes ventricular tachycardia. Hydrofluoric acid has widespread usage in industry (Kirkpatrick et al., 1995).

There are various methods for decontaminating and treating HF dermal Burns (Upfal and Doyle, 1990; el Saadi et al., 1989; Matsuno, 1996; Bentur et al., 1993; Greco et al., 1988; Trevino et al., 1983; Browne, 1974; Wetherhold and Sheperd, 1965). Concentrated HF (40–70%, anhydrous) rapidly produces painful lesions (Griffith, 1987), requiring that decontamination and treatment be undertaken immediately. In spite of early water decontamination followed by repeated topical calcium gluconate inunction or subcutaneous injection, development of burns often cannot be prevented. The risk of systemic and sometimes fatal HF toxicity is greatest with concentrated HF splashes (Tepperman, 1980).

Dilute HF has sometimes been successfully decontaminated with water followed by topical application of calcium gluconate gel. The difficulty in such cases is perception of the need to immediately undertake these measures in the absence of pain, which may be delayed in onset (Griffith, 1987; Saada et al., 1995; Henry and Hla, 1992). The duration of the HF contact with the tissues may be prolonged in such situations. With dermal exposure to either dilute or concentrated HF, surgical debridement, excision, or even amputation of necrotic areas may be required in certain cases (Chick and Borah, 1990; Buckingham, 1988; Julie et al., 1987; Carney et al., 1974).

Immediate water decontamination, especially with concentrated HF skin/eye splashes, is not sufficient to prevent tissue injury and systemic toxicity in many cases.

90.4 DISCUSSION

Water decontamination has the following proposed mechanism of action (Bromberg et al., 1965): (1) dilution of the chemical agent; (2) rinsing off the chemical agent; (3) decreasing the rate of the chemical reaction; (4) decreasing tissue metabolism and therefore the inflammatory reaction; (5) minimizing the hygroscopic effects of chemicals that produce them; and (6) restoring normal skin pH in acid and alkali burns.

The ANSI Z358.1-1998 standard is a consensus standard for emergency water decontamination equipment for the skin and eyes (Bollas and Coffey, 1998). It specifies that emergency showers should deliver a pattern of flushing solution 20 in. (50.8 cm) across with a flow rate of 20 gallons (75.7 L) per minute and a velocity sufficiently low as not to cause injury to the user (Bollas and Coffey, 1998). For eyewash stations, a 15 min uninterrupted water supply must be available and plumbed units should deliver between 2.0 and 3.5 gallons (7.5–13.25 L) per minute (Bollas and Coffey, 1998). These emergency decontamination stations should be clearly marked and should take a chemical-exposed worker no more than 10 s to reach (Bollas and Coffey, 1998).

For eye decontamination, it has been said "The ideal flushing solution is a sterile, isotonic, preserved, physiologically balanced solution" (Hurley, 1998). However, "At a minimum, flushing fluid should be clean and nontoxic," which would include potable water (Hurley, 1998). There remains the problem of disposal of water used for skin/eye decontamination of chemical splashes, particularly whether a chemical can be discharged in the sanitary system even in a very dilute concentration (Bollas and Coffey, 1998). In some cases, it may be necessary to install a special holding tank for runoff water from emergency eyewashes and showers and to dispose of the decontamination water as hazardous waste (Bollas and Coffey, 1998).

In a frequently cited review, Jelenko (1974) noted that chemical agents do not "burn" in the classic sense of tissue destruction by heat. Rather, they act by coagulating protein through oxidation, reduction, salt formation, corrosion, protoplasmic poisoning, metabolic competition or inhibition, desiccation, or vesicant activity and its resultant ischemia. Although neutralization has been said not to be as efficacious as water decontamination for acid and alkali exposures (Bromberg et al., 1965), Lewis (1959) recommended initial copious water decontamination followed by neutralization and then a second decontamination with copious amounts of water.

Psychosocial consequences are often overlooked in patients with chemical burns, especially those involving the head, face, eyes, and neck. Rumsey et al. (2003) found that a considerable portion of patients with disfiguring conditions, including burns, had psychosocial difficulties including increased anxiety levels, depression, social anxiety, social avoidance, and reduced life quality.

90.5 CONCLUSION

From this review, it is clear that while chemical burn injuries represent a small portion of total burn injuries, their human and economic impact is significant. Although immediate water decontamination has generally been shown to decrease the severity of chemical skin/eye burns, it is also obvious that it does not prevent such burns from developing, nor does it always prevent the need for lost work time, hospitalization, burn center/unit admission, the requirement for surgical treatment, and sequelae. Significant sequelae and death can occur following chemical splashes, even when water decontamination is done on a timely basis.

Given a renewed interest in neutralization measures, decontamination solutions that are sterile, chelating, polyvalent (bind a wide variety of chemicals/chemical groups), amphoteric (bind opposed chemical groups such as acids/bases, oxidizers/reducing agents, etc.), nontoxic, hypertonic (to help prevent skin/cornea penetration), and water-soluble (so that beneficial diluting and rinsing effects of water are not lost) should be critically evaluated by those concerned with initial decontamination of skin/eye chemical splashes. Comparative, blinded, controlled studies of various eye/skin decontamination solutions, including water, are needed.

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91 Chemical Substances and Contact Allergy: 244 Substances Ranked According to Allergenic Potency

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91.1 INTRODUCTION

Allergic contact dermatitis is the prototype clinical manifestation of a type IV delayed-type inflammatory reaction arising from percutaneous contact with putative allergens. The clinical picture is usually a quite typical eczema that exhibits spreading and pruritus. Suspected contact dermatitis requires detailed evaluation of the patient's history and a meticulous physical examination. A high index of suspicion and a relevant investigation is required. Reliable and sensitive animal test methods can identify contact allergens and are thus an invaluable tool for the prediction of contact allergenic properties for those substances that will be placed on the market as "new substances."

To discuss and decide on the potency ranking of 244 substances, clinical and experimental data on humans and results of animal tests as documented in the literature were carefully collected and evaluated. These documents have been published in German. To make the data available to the international scientific community, the experts decided to publish in English an overview on the ranking and a summary of these data. These data present a useful database of the chemical structures identified as triggering with different potency an immunological process that leads to contact sensitization or to contact allergic cross-reactions. They may also be useful in identifying additional chemicals with contact allergenic properties on the basis of structure–activity relationships (SAR) or in refining existing rules for the determination of an allergenic potential on the basis of structural alerts.

91.2 METHODS

At the department Assessment of Chemicals of the Federal Health Agency (BGA) in Germany (since 1996, the Federal Institute for Health Protection of Consumers and Veterinary Medicine [BgVV] and since November 2002, the Federal Institute for Risk Assessment [BfR]), an expert group on skin sensitization (group "Allergen List") was established in April 1985, and 34 meetings were held till June 2001. A total of 30 experts participated (the authors of this publication and the members listed in the Addendum). The aim of this project was to bring together a broad range of expertise to collect and evaluate data from the literature on substances with documented contact allergenic properties in humans and in animal experiments. The major aim was to evaluate whether potency ranking of known or suspected contact allergens is feasible and to develop respective criteria, if possible. For that, the available data of known or suspected contact allergens were collected and compared to evaluate differences with respect to potency. Although there was at that time a general scientific agreement on the differences with respect to potency of contact allergens, a ranking using a systematic approach had not yet been pursued.

TABLE 91.1
Definition of Categories

Category	Definition	Remarks
Category A	Significant contact allergen because of 1. proven strong contact allergenic effect in humans after short or almost negligible exposure taking into account existing animal data, 2. frequently proven contact allergenic effect in humans.	Data on humans demonstrate that in larger collectives, 1% or more of the patients react positive and that several independent case studies and experimental data on humans are available.
Category B	Solid-based indication for contact allergenic effects because of 1. less frequently proven contact allergenic effect in humans taking into account existing positive animal data, 2. the capacity of substances to induce cross-reactions in humans without being a significant allergen itself.	Data on humans demonstrate that in collectives less than 1% of the patients react positive and that independent case studies and experimental data on humans are available.
Category C	Insignificant contact allergen or questionable contact allergenic effect because of: 1. rarely proven contact allergenic effect in humans, 2. doubtful effect in humans; no or nonappropriate animal data, 3. no data on humans but positive animal data.	Data on humans include isolated positive test results and isolated case studies and experimental data.

In a first step, two members of the group collected and published data on 956 substances with published evidence of contact allergenic properties, however, without evaluating the potency of the contact allergenic properties of these substances. For each of these substances, the commonly used name, the chemical structure, Chemical Abstract Service (CAS) number, animal data, human data, test concentration for patch testing, occurrence, and references for the respective substance were listed (Klaschka and Vossmann, 1994).

A thorough evaluation of these 956 substances confirmed the need to categorize the substances according to their allergenic potency and their relevance to human health. As a second step, the ranking criteria shown in Table 91.1 were developed after very intense and long-lasting discussions. To select the substances for further evaluation within the newly developed ranking system, the dermatologists/allergologists selected the most relevant substances on the basis of their practical experience and the representatives of the chemical industry selected the substances produced by companies that are members of the German Chemical Manufacturers' Association (VCI). Eventually, certain substances were included that were not produced by the chemical industry but were considered to be of relevance to consumers. Other toxicological properties, such as general toxicity and carcinogenic or mutagenic properties, were not considered. The occurrence and use of the substances were documented as described in the literature. With very few exceptions, pharmaceuticals and pesticides were not included.

Out of these 956 substances, 244 substances were further evaluated, and the evaluations were published as a loose-leaf book (Kayser and Schlede, 2001) in German. In these documents, the main substance name is given in German. A list of all synonyms including the substance names in English is also available. For each substance, a data sheet was compiled that contained the following information: commonly used substance name, CAS number, synonyms (including substance name according to International Union of Pure and Applied Chemistry (IUPAC) nomenclature), empirical formula, chemical structure, occurrence/use, experience in humans (clinical data and experimental studies), animal data, conclusions, remarks, and references. A complete list of references was not listed in all cases. The only references that were given were those that allowed a judgment on the contact allergenic properties of a substance. Primarily on the basis of expert judgment in combination with careful literature evaluations, these substances were allocated into one of the defined categories.

The term "chemical substance" was defined as follows: technically produced chemicals as well as chemically defined single ingredients of natural products.

91.3 RESULTS

Ninety-eight substances were listed in category A (see Appendix A), 77 substances in category B (see Appendix B), and 69 substances in category C (see Appendix C). Then the structural formula, CAS number, and occurrence/use of the substances are listed, followed by an overview on the data in humans and animals.

Clinical data include results from patch testing of exposed subjects, i.e., case reports, selected collectives (such as eczema patients), or both. A criterion for listing a substance in category A ("significant contact allergen") was that in the testing of larger collectives 1% or more of the patients reacted positively and that several independent case studies, positive experimental data on humans, or both were available.

Listing in category B ("solid-based indication for a contact allergenic potential") was based on data in humans that demonstrated that in larger collectives less than 1% of the patients reacted positive. Furthermore, independent case studies, experimental data on humans, or both were available. In addition, in this category substances are also included that by themselves are not solid-based contact allergens but have the capacity to lead to cross-reactions in humans.

The evidence of cross-reactions was found not only for substances in category B but also for substances in categories A and C. However, for the substances listed in these categories, the ability to elicit a cross-reaction did not determine the strength of allergenic potency. For cross-reacting substances listed in category C, this specific property was considered to be negligible.

For substances listed in category C ("contact allergen with insignificant or questionable contact allergenic potential"), only isolated positive patch test results in humans and isolated case studies with or without positive animal data or positive animal data only were documented.

For some substances, published experimental data in humans were obtained with various test methods, the most prominent being the maximization test and the repeated insult patch test (RIPT). In contrast, animal data have been obtained by using a large variety of test methods. All available animal data were included in the evaluation, not only data from the currently most often used and standardized guinea pig maximization test according to Magnusson and Kligman (1969) and the Buehler Test (1965) or the recently adopted and validated local lymph node assay (Kimber et al., 1998).

Table 91.2 gives an overview of the use/occurrence of the substances. Of the 244 substances, 105 (43%) are used as ingredients in consumer products, and 67 of these 105 substances are used only as ingredients in cosmetics; 86 (35%) out of the total 244 substances are used only as industrial chemicals, 32 (13%) have other uses, such as dental fillings, and 59 (24%) are used in both nonindustrial and industrial use.

Table 91.3 gives an overview of the data recorded in the specific categories and the sum of these data. For 231 (95%)

TABLE 91.2
Use of Substances in Various Areas

	Consumer Use				Industrial Use					Other Use[a]		
Category	Household Only	Cosmetic Only	Household/ Cosmetic	Industrial Use Only	Household	Cosmetic	Household/ Cosmetic	Other	Other Use Only[a]	Household	Cosmetic	Household/ Cosmetic
A (n = 98)	10	20	11	31	7	10	7	18	17	6	8	6
B (n = 77)	6	19	7	27	5	11	1	16	8	2	10	4
C (n = 69)	1	28	3	28	1	17	1	25	7	1	21	2
Sum (n = 244)	17	67	21	86	13	38	9	59	32	9	39	12

[a] Other use, i.e., as dental fillings, flavoring substances, substances used for research and development, pharmaceuticals, and ingredients of pharmaceuticals.

TABLE 91.3
Summary of Human and Animal Data on the 244 Evaluated Substances

	Category A (n = 98)	Category B (n = 77)	Category C (n = 69)	Sum (n = 244)
Human data				
Clinical data	**97 (99%)**	**76 (99%)**	**58 (84%)**	**231 (95%)**
Cross-reaction	53 (55%)	36 (47%)	7 (12%)	96 (42%)
Experimental data	**33 (34%)**	**23 (30%)**	**21 (36%)**	**77 (32%)**
Experimental data only[a]	1	—	—	1
Positive	24 (72%)	5 (22%)	3 (14%)	32 (42%)
Positive/negative	4 (12%)	4 (17%)	3 (14%)	11 (14%)
Negative	5 (15%)	14 (61%)	15 (71%)	34 (44%)
Animal data	**91 (93%)**	**71 (92%)**	**54 (78%)**	**215 (88%)**
Animal data only[a]	—	1	11	12
Positive	71 (78%)	31 (44%)	21 (39%)	123 (57%)
Positive/negative	19 (21%)	36 (51%)	20 (37%)	75 (35%)
Negative	1 (1%)	3 (4%)	13 (24%)	17 (8%)

Note: Bold numbers refer to absolute numbers.

[a] Positive data only.

TABLE 91.4
Comparison between Clinical Data and Animal Data

Clinical human data	231 (100%)	
Animal data:	215 (100%)	
Positive	123 (57%)	} 92%
Positive/negative	75 (35%)	
Negative	17 (8%)	

TABLE 91.5
Comparison between Experimental Data on Humans and Animal Data

		Animal Data		
Category	Experimental Human Data	Positive	Positive/ Negative	Negative
A	Positive (24)	16	7	1
	Positive/negative (4)	2	2	0
	Negative (5)	3[a]	1	0
B	Positive (5)	3	2	0
	Positive/negative (4)	0	4	0
	Negative (14)	3	10	1
C	Positive (3)	2[a]	0	0
	Positive/negative (3)	0[a]	1	1
	Negative (15)	1	8	6

[a] No data on 1 substance.

of the 244 substances, clinical data were available in the literature. Cross-reactions have been demonstrated for 96 out of these 231 substances (42%). Experimental data in humans were found for 77 substances (32%) yielding results that ranged from positive to negative. As expected, the highest number of positive experimental data in humans were found in category A (72%) and substantially lower in categories B and C (22% and 14%, respectively). For the overwhelming majority of the substances (215 of the 244 substances, i.e., 88%), animal data were available. In 123 of these 215 (57%) substances (categories A, B, and C), animal data were consistently positive. Eleven of the total 244 substances were categorized only on the basis of positive animal data with no data available for humans (category C). A combination of positive and positive/negative animal data yielded a high percentage of 92%. In general, a combination of positive/negative animal data was obtained when substances were examined using "old" and nonstandardized test methods, inappropriate test concentrations, or both. Another reason for the discrepancy in results was a difference in the sensitivity of test methods used. For only 17 (8%) of the substances, the available animal data showed negative results only (Table 91.4). For these substances, the results obtained with human data were used for categorization (predominantly category C). In summary, it can be concluded that results obtained with animal data are reliable and sensitive indicators for the prediction of a contact allergenic potential in humans.

A comparison between experimental human data and animal data shown in Table 91.5 demonstrates a more complex situation. In the majority, positive human experimental data correlate with positive and positive/negative animal data. There are—with one exception—no negative data in animals compared to positive experimental data on humans.

91.4 DISCUSSION

At the start of the work of the expert group "Allergen List" in 1985, there was, and still is, hardly any detailed documentation of contact allergens with different potencies based on clinical and experimental data on humans and animals. The work of the expert group with the development of a ranking system and successful categorization of substances with regard to the skin-sensitizing potential and potency further strengthens the general need not only for a potency ranking of chemicals for dermatologists (Wilkinson et al., 2002) but also for regulatory purposes in the framework of preventive health protection. The World Health Organization (1997) also recommends the ranking of substances according to their allergenic potency. The Organisation for Economic Co-Operation and Development (OECD, 1999, 2001) recommends a specific category for strong contact sensitizers, and also national scientific committees such as the Commission of the Deutsche Forschungsgemeinschaft for the Investigation of Health Hazards of Chemical Compounds in the Work Area have developed very recently criteria based on the inherent allergenic properties and the allergenic potency by using available human and animal data (Schnuch et al., 2002).

The high percentage of substances for which cross-reactions in patients are documented (42%) raises concern. So far, definite cross-reactions among structurally related substances in patch-tested patients can only be anticipated if relevant exposure can be excluded. Otherwise these reactions may also be regarded as concomitant reactions based on cosensitization. Therefore, it greatly depends on the experience of the dermatologist and the knowledge about exposure to evaluate cross-reactions in a patient. The presently used animal test methods are useful in studying cross-reactions and have therefore been used in several cases for this purpose. However, these methods were not specifically developed to address this aspect.

A safety assessment and also the risk assessment for existing substances already placed on the market are best made by using data on all aspects, i.e., human and animal data. For newly developed chemicals that will be placed on the market as new substances, only animal data with highly standardized tests are available (Schlede et al., 2001). A positive animal test can be judged as a reliable indicator for a contact allergenic potential in humans. This aspect is further strengthened by a comparison between experimental human data and animal data demonstrating that results obtained in

animals are more reliable indicators for the prediction of contact allergenic properties than human experimental data. However, it should be taken into account that published data on experimental human testing are limited in most cases to older studies with insufficient experimental design, limited documentation, or both. Because of ethical reasons, the test concentrations used were in some cases not maximized, but rather exposure relevant. For example, the human RIPT nowadays is performed to confirm the absence of skin sensitization activity of formulations under specific conditions of use (Kimber et al., 2001), and not to study a skin sensitization potential in general.

The data used for this English presentation are published in German in data sheets for each of the 244 substances as a loose-leaf book (Kayser and Schlede, 2001) and on the Internet (BfR, 2003; DIMDI, 2003). These data sheets are the basis for the information as shown in Appendices A through C, which provide an excellent overview and a compilation of chemical structures and information on the existence of human and animal data associated with contact allergy. They are also a valuable tool for improving and refining SAR models and rules in expert systems for the prediction of contact allergenic properties of chemicals. For example, these data were already used to amend specific "structural alerts" for the prediction of contact allergenic properties of chemicals used in an existing expert system (Barratt and Langowski, 1999, 2000). This approach was further validated by using additionally a regulatory database of chemicals notified in the European Union (Zinke et al., 2002; Gerner et al., 2003). Such expert systems are also of importance in minimizing the use of animals for the assessment of a probable contact allergenic hazard.

The work as presented here is a basis for the ongoing discussion in the scientific and regulatory community to develop internationally harmonized criteria for the categorization of contact allergens with different potencies.

ADDENDUM

Besides the authors of this publication, the group "Allergen List" consisted of the following members:

Representatives from universities and specialists in dermatology and allergology: Prof. Dr. F. Klaschka and Dr. D. Voßmann, Free University, Klinikum Steglitz, Berlin; Prof. Dr. K.-H. Schulz, University of Hamburg; Prof. Dr. H. Ippen, University of Göttingen; Prof. Dr. G. Richter, University of Dresden; and Dr. A. Rothe, Berlin (all based in Germany).

Representatives from the chemical industry: Prof. Dr. E. Löser, Bayer AG, Wuppertal; Dr. W. Matthies and Dr. N. Banduhn, Henkel KGaA, Düsseldorf; Dr. R. Snethlage and Dr. D. Bury, Hoechst AG, Frankfurt/M, Dr. H. Kuhn and Dr. O. J. Grundler, BASF AG, Ludwigshafen; and Dr. U. Jensch, Clariant GmbH, Sulzbach im Taunus (all based in Germany).

Representatives from federal agencies: Dr. R. Roll, Dr. W. Diener, and Dr. R. Eppler, Federal Institute for Risk Assessment (BfR), Berlin and Federal Institute for Health Protection of Consumers and Veterinary Medicine (BgVV), Berlin; Prof. Dr. B. Schlatterer, Federal Environmental Agency (UBA), Berlin; and Dr. S. Darschnik and Dr. I. Hoepfner, Federal Institute for Occupational Safety and Health (BAuA), Dortmund (all based in Germany).

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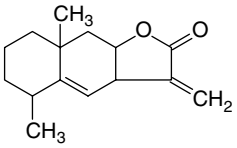
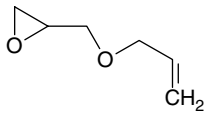
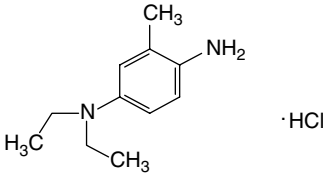
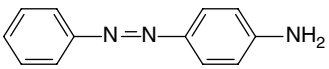
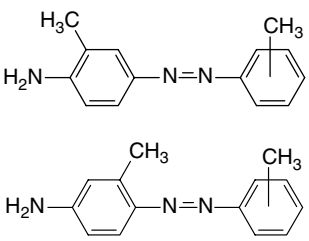
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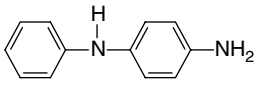
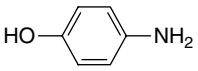
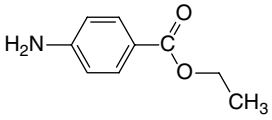
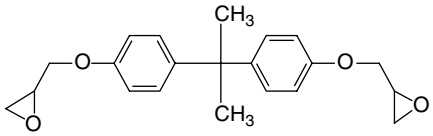
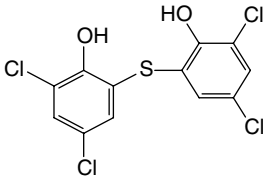
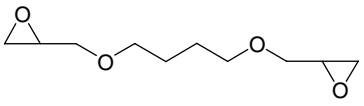
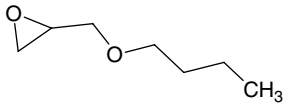
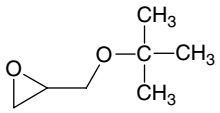
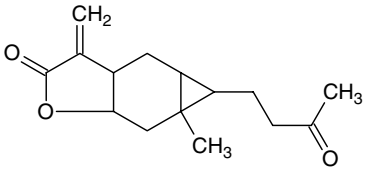
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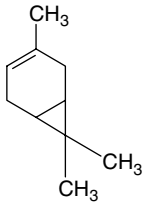
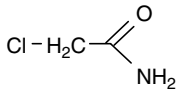
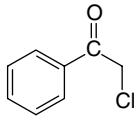
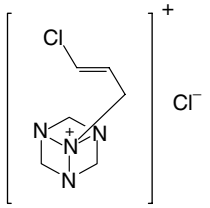
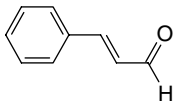
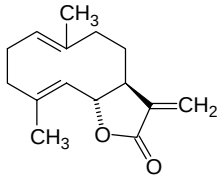
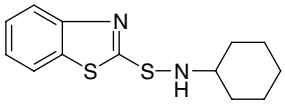
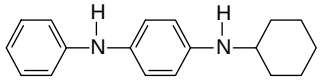
Appendix A: Summary of Data on Substances Listed in Category A

Substance/Structure	CAS No.	Use			Human Data			Animal Data	
		Consumer Use	Industrial Use	Other Use	Clinical	Cross Reaction	Experimental	Positive	Negative
Alantolactone[a,b] 	546-43-0	h/-		x	x	x		x	
Allyl glycidyl ether 	106-92-3	-/-	x		x	x		n.d.	
2-Amino-5-diethylaminotoluene hydrochloride 	2051-79-8	h/-	x		x	x	p	x	
p-Aminoazobenzene 	60-09-3	-/-	x		x	x		x	
p-Aminoazotoluene 	41576-40-3	h/-	x		x	x		x	

Appendix A (Continued)

Substance/Structure	CAS No.	Use			Human Data			Animal Data	
		Consumer Use	Industrial Use	Other Use	Clinical	Cross Reaction	Experimental	Positive	Negative
4-Aminodiphenylamine 	101-54-2	-/c	x		x	x		x	
p-Aminophenol 	123-30-8	-/c	x		x	x		x	x
Benzocaine[b] 	94-09-7	-/c		x	x	x	p/n	x	
Bisphenol A diglycidyl ether 	1675-54-3	-/-	x		x	x		x	
Bithionol[b] 	97-18-7	-/-	x	x	x	x	p/n	x	
1,4-Butanediol diglycidyl ether 	2425-79-8	-/-	x		x	x		x	
n-Butyl glycidyl ether 	2426-08-6	-/-	x		x	x	p	x	
t-Butyl glycidyl ether 	7665-72-7	-/-	x				p	x	x
Carabrone[a,b] 	1748-81-8	-/-		x	x	x		x	

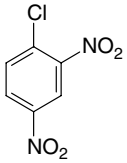
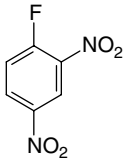
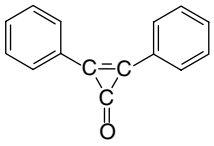
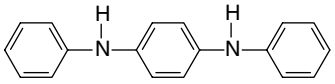
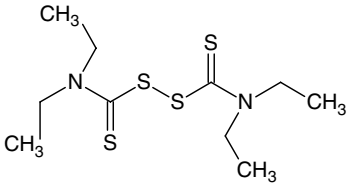
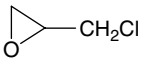
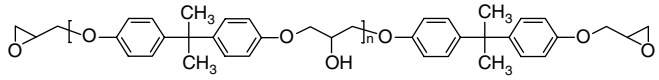
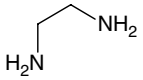
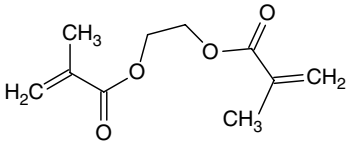
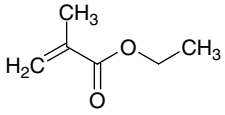
Appendix A (Continued)

Substance/Structure	CAS No.	Use			Human Data			Animal Data	
		Consumer Use	Industrial Use	Other Use	Clinical	Cross Reaction	Experimental	Positive	Negative
Δ^3-Carene and its oxidation products 	13466-78-9	h/-	x		x			x	
Chloroacetamide 	79-07-2	h/c	x		x	x	p	x	x
2-Chloroacetophenone[b] 	532-27-4	-/-		x	x			x	
N-(3-Chloroallyl) hexaminium chloride 	4080-31-3	h/c	x		x			x	x
Cinnamaldehyde[a] 	104-55-2	h/c			x		p/n	x	
Cobalt and its salts	7440-48-4	-/-	x		x			x	
Colophony[a,b]	8050-09-7	h/c	x	x	x			x	
Costunolide[a,b] 	553-21-9	h/c		x	x	x	p		x
N-Cyclohexylbenzothiazylsulfenamide 	95-33-0	-/-	x		x			n.d.	
N-Cyclohexyl-*N*′-phenyl-*p*-phenylenediamine 	101-87-1	-/-	x		x	x		x	

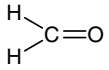
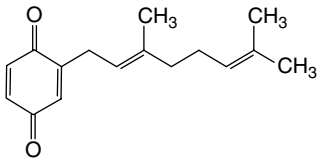
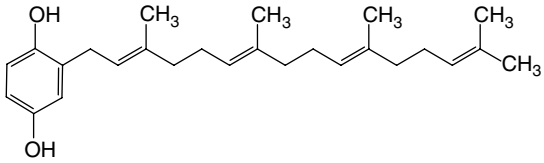
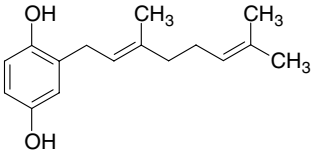
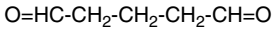
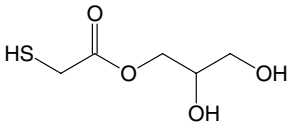
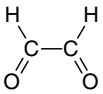
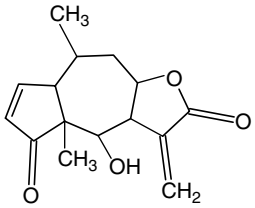
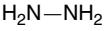
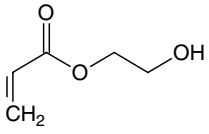
Appendix A (Continued)

Substance/Structure	CAS No.	Use			Human Data			Animal Data	
		Consumer Use	Industrial Use	Other Use	Clinical	Cross Reaction	Experimental	Positive	Negative
Desoxylapachol	3568-90-9	-/-	x		x			x	
4,4′-Diaminodiphenylmethane	101-77-9	-/-	x		x	x		x	
4,5-Dichloro-2-methylisothiazolinone	26542-23-4	h/c	x		x	x		x	
Dichlorophen[b]	97-23-4	-/c	x	x	x		n	x	
Dicyclopentamethylene thiuramdisulfide	94-37-1	-/-	x		x	x		n.d.	
N,*N*-Diethylamino-1,4-phenylenediamine (base)[b]	93-05-0	h/c	x	x	x	x		x	
Diethylenetriamine	111-40-0	-/-	x		x	x	p	x	x
R-3,4-Dimethoxydalbergione	3755-64-4	-/-	x		x			x	

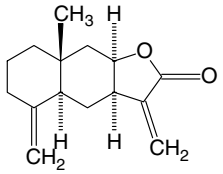
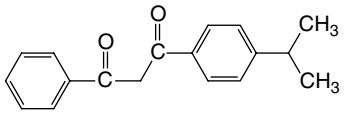
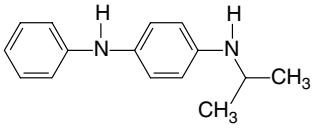
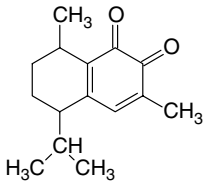
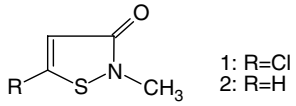
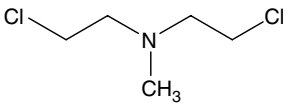
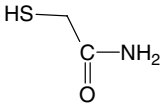
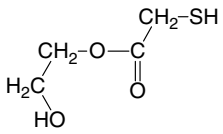
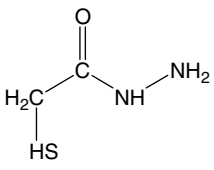
Appendix A (Continued)

Substance/Structure	CAS No.	Use			Human Data			Animal Data	
		Consumer Use	Industrial Use	Other Use	Clinical	Cross Reaction	Experimental	Positive	Negative
2,4-Dinitrochlorobenzene[b] 	97-00-7	-/-	x	x	x	x	p	x	
2,4-Dinitrofluorobenzene[b] 	70-34-8	-/-		x	x	x		x	
Diphencyprone[b] 	886-38-4	-/-		x	x			x	
N,*N*′-Diphenyl-*p*-phenylenediamine 	74-31-7	-/-	x		x	x		x	
Disulfiram[b] 	97-77-8	h/-	x	x	x	x		n.d.	
Epichlorohydrin 	106-89-8	-/-	x		x			x	
Epoxy resins 	25068-38-6	-/-	x		x			x	x
Ethylenediamine[b] 	107-15-3	-/-	x	x	x	x	p	x	x
Ethylene glycol dimethacrylate[b] 	97-90-5	-/c	x	x	x	x		x	x
Ethyl methacrylate[b] 	97-63-2	-/c	x	x	x			x	x

Appendix A (Continued)

Substance/Structure	CAS No.	Use			Human Data			Animal Data	
		Consumer Use	Industrial Use	Other Use	Clinical	Cross Reaction	Experimental	Positive	Negative
Formaldehyde[b] 	50-00-0	h/c	x	x	x			x	
Geranylbenzoquinone[a,b] 	61977-06-8	-/-		x	x			x	
Geranylgeranylhydroquinone[a,b] 	39703-09-8	-/-		x	x	x		x	
Geranylhydroquinone[a,b] 	10457-66-6	-/-		x	x			x	
Glutaraldehyde[b] 	111-30-8	h/-	x	x	x		p/n	x	x
Glyceryl monothioglycolate 	30618-84-9	-/c			x			n.d.	
Glyoxal[b] 	107-22-2	h/-		x	x		p	x	
Helenalin[a,b] 	6754-13-8	-/-		x	x	x		x	
Hydrazine and its salts[b] 	302-01-2	-/-	x		x	x	p	n.d.	
2-Hydroxyethyl acrylate 	818-61-1	-/-	x		x			x	x

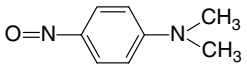
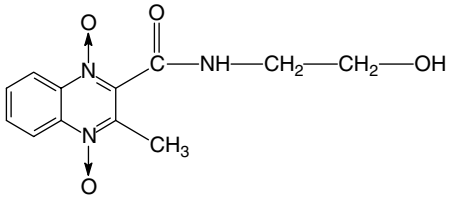
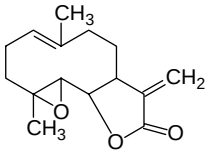
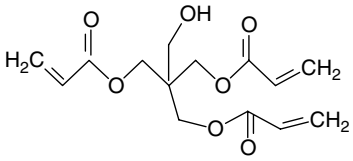
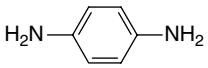
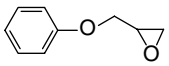
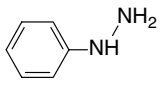
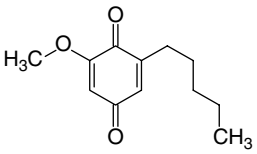
Appendix A (Continued)

Substance/Structure	CAS No.	Use			Human Data			Animal Data	
		Consumer Use	Industrial Use	Other Use	Clinical	Cross Reaction	Experimental	Positive	Negative
Isoalantolactone[a,b] 	470-17-7	-/-		x	x			x	
4-Isopropyldibenzoylmethane 	63250-25-9	-/c			x			x	
N-Isopropyl-*N′*-phenyl-*p*-phenylenediamine 	101-72-4	-/-	x		x	x		x	
Laurel oil[a,b]	8002-41-3	h/c		x	x		n	x	x
Mansonone A[a] 	7715-94-8	-/-	x		x			x	
MCI/MI 	55965-84-9	h/c	x		x			x	
Mechloroethamine[b] 	51-75-2	-/-		x	x			x	
Mercaptoacetamide[b] 	758-08-7	-/-		x	x	x	p	x	
Mercaptoacetic acid-2-hydroxyethyl ester 	7380-58-7	-/c			x	x		x	
Mercaptoacetic acid hydrazide 	760-30-5	-/c			x	x		x	

Appendix A (Continued)

Substance/Structure	CAS No.	Use			Human Data			Animal Data	
		Consumer Use	Industrial Use	Other Use	Clinical	Cross Reaction	Experimental	Positive	Negative
2-Mercaptobenzothiazole	149-30-4	-/-	x		x	x		x	x
Mercury compounds, organic[b]	22967-92-6	-/c		x	x	x	p	x	
Methyl methacrylate[b]	80-62-6	-/c	x	x	x		p/n	x	x
N,N′-Methylene-*bis*(5-methyloxazolidine)	66204-44-2	-/-	x		x	x		x	
α-Methylene-γ-butyrolactone[a]	547-65-9	-/-		x	x			x	
N-Methylolchloroacetamide	2832-19-1	-/-	x		x	x		x	
Moschus-Ambrette[b]	83-66-9	h/c		x	x		n	x	
Naphthol AS	92-77-3	-/-	x		x			x	
Nickel and its salts	7786-81-4	h/-	x		x		p	x	x
Nitramine[b]	479-45-8	-/-		x	x	x		x	
p-Nitrobenzylbromide	100-11-8	-/-	x		x		p	x	
p-Nitro-ω-bromoacetophenone	99-81-0	-/-	x		x		n	n.d.	
Nitro-*p*-phenylenediamine	5307-14-2	-/c			x			x	

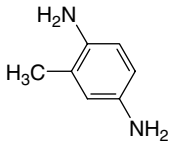
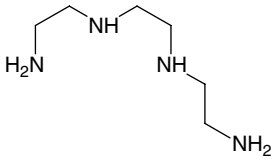
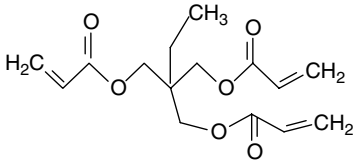
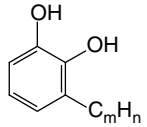
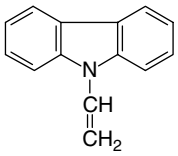
Appendix A (Continued)

Substance/Structure	CAS No.	Use			Human Data			Animal Data	
		Consumer Use	Industrial Use	Other Use	Clinical	Cross Reaction	Experimental	Positive	Negative
p-Nitrosodimethylaniline 	183-89-6	-/-	x		x	x	p	x	
Oil of turpentine[a,b]	8006-64-2	h/-	x	x	x	x	p	x	
Olaquindox[b] 	23696-28-8	-/-		x	x			x	x
Parthenolide[a,b] 	20554-84-1	-/-		x	x	x		x	
Pentaerythrit triacrylate[b] 	3524-68-3	-/-	x	x	x	x	p	x	
Peruvian balsam[a,b]	8007-00-9	-/c		x	x			x	
p-Phenylenediamine 	106-50-3	-/c	x		x	x	p	x	
Phenyl glycidyl ether 	122-60-1	-/-	x		x	x		x	
Phenylhydrazine 	100-63-0	-/-	x		x	x		x	
Potassium dichromate (VI)	7778-50-9	-/-	x		x		p	x	x
Primin[a] 	15121-94-5	-/-		x	x			x	

Appendix A (Continued)

Substance/Structure	CAS No.	Use			Human Data			Animal Data	
		Consumer Use	Industrial Use	Other Use	Clinical	Cross Reaction	Experimental	Positive	Negative
Propolis[a]	85665-41-4	-/c		x	x			x	
1: R′=R″=H, R=X^1 (Caffeic acid 3-methyl-2-butenyl ester) 2: R′=R″=H, R=X^2 3: R′=R″=H, R=X^3 4: R′=R″=H, R=X^4 5: R′=R″=H, R=-OH 6: R′=R″=H, R=-CH_2-C_6H_5 7: R′=R″=H, R=-CH_2-CH_2-C_6H_5 8: R′=-CH_3, R"=H, R=-CH_2-C_6H_5									
Quinoline yellow spirit soluble	8003-22-3	-/c	x		x		p	x	x
Squaric acid dibutyl ester/squaric acid diethyl ester[b] R=C_2H_5: Ethyl ester R=C_4H_9: Butyl ester	5231-87-8	-/-		x	x			x	
Tetrachlorosalicylanilide	1154-59-2	h/c			x	x		x	
Tetramethylthiuram monosulfide	97-74-5	-/-	x		x		p	x	
3-Thioglycerol	96-27-5	-/c			x		p	x	x
Thiram[b]	137-26-8	-/c	x	x	x	x	p	x	

Appendix A (Continued)

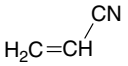
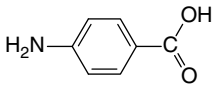
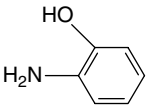
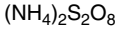
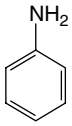
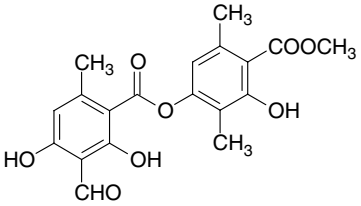
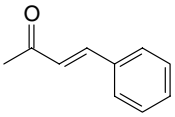
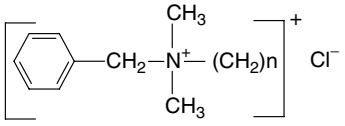
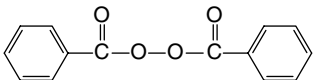
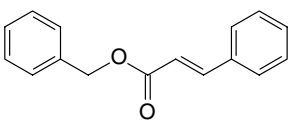
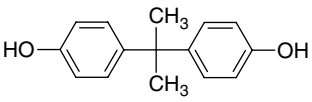
Substance/Structure	CAS No.	Use			Human Data			Animal Data	
		Consumer Use	Industrial Use	Other Use	Clinical	Cross Reaction	Experimental	Positive	Negative
p-Toluylenediamine and its salts 	95-70-5	-/c	x		x	x	n	x	
Triethylenetetramine[b] 	112-24-3	-/-	x	x	x	x		x	
Trimethylolpropane triacrylate 	15625-89-5	-/-	x	x	x			x	
Urushiol[a]  *m*=13–17, *n*=27–35	53237-59-5	h/-		x	x			x	
Vinylcarbazol 	1484-13-5	-/-	x	x	x			x	

Note: h: household; c: cosmetics; p: positive; n: negative; n.d.: no data.

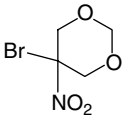
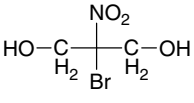
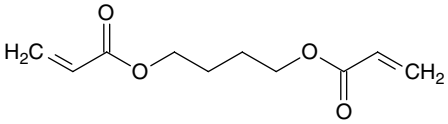
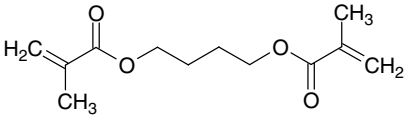
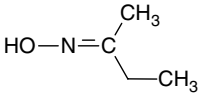
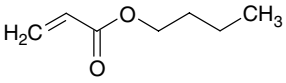
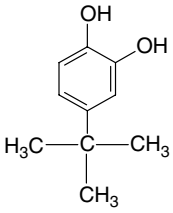
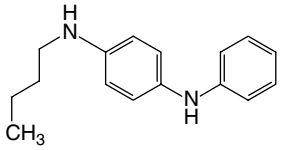
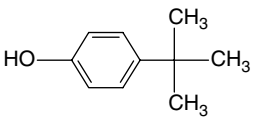
[a] Natural constituent of plants.

[b] Other use, i.e., as dental fillings, flavoring substances, substances used for research and development, pharmaceuticals, and ingredients of pharmaceuticals.

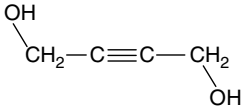
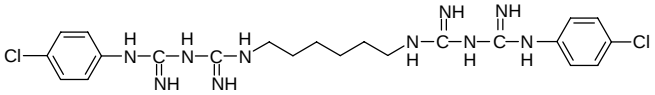
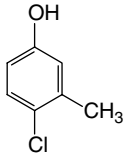
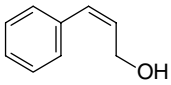
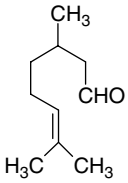
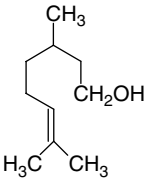
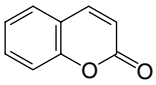
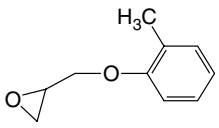
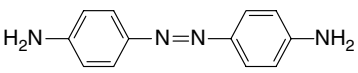
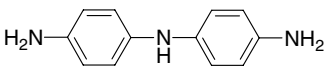
Appendix B: Summary of Data on Substances Listed in Category B

Substance/Structure	CAS No.	Use			Human Data			Animal Data	
		Consumer Use	Industrial Use	Other Use	Clinical	Cross Reaction	Experimental	Positive	Negative
Acrylonitrile 	107-13-1	-/-	x		x			x	
Amalgam[b]	8049-95-2	-/-		x	x			n.d.	
p-Aminobenzoic acid 	150-13-0	-/c	x		x	x	n	x	x
o-Aminophenol 	95-55-6	-/c	x		x	x		x	x
Ammonium persulfate 	7727-54-0	-/c	x		x			x	
Aniline 	62-53-3	-/-	x		x	x	p	x	x
Atranorin[a] 	479-20-9	-/c	x	x	x			x	
Benzalacetone[b] 	122-57-6	-/c		x	x		p	x	
Benzalkonium chloride[b] 	8001-54-5	h/c		x	x		n	x	x
Benzoyl peroxide[b] 	94-36-0	-/-	x	x	x		p	x	
Benzyl cinnamate[a] 	103-41-3	-/c		x	x		n	x	x
Bisphenol A 	80-05-7	-/-	x		x	x		x	x

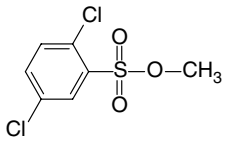
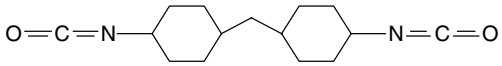
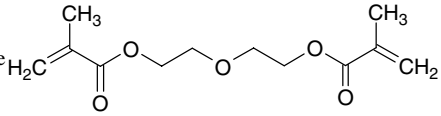
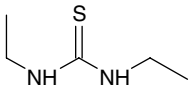
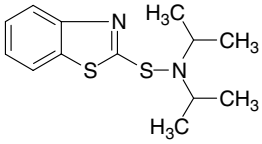
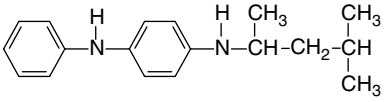
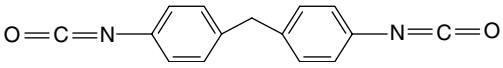
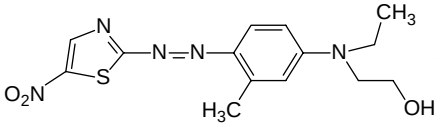
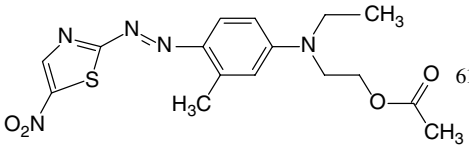
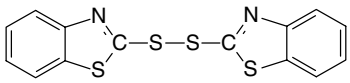
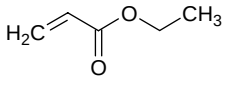
Appendix B (Continued)

Substance/Structure	CAS No.	Use			Human Data			Animal Data	
		Consumer Use	Industrial Use	Other Use	Clinical	Cross Reaction	Experimental	Positive	Negative
Bronidox 	30007-47-7	-/c			x			x	
Bronopol 	52-51-7	-/c	x	x	x		p/n	x	x
1,4-Butanediol diacrylate 	1070-70-8	-/-		x	x			x	x
1,4-Butanediol dimethacrylate 	2082-81-7	-/-	x		x	x		x	x
Butanone oxime 	96-29-7	-/-	x		n.d.			x	
n-Butyl acrylate 	141-32-2	-/-		x	x			x	
p-tert-Butylcatechol 	98-29-3	-/-	x	x	x	x			x
N-Butyl-*p*-aminodiphenylamine 	15464-99-0	-/-	x		x	x		x	
p-tert-Butylphenol 	98-54-4	-/-	x		x	x		x	x

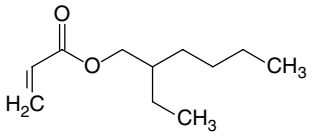
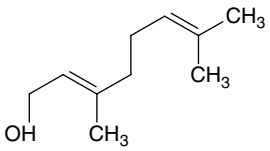
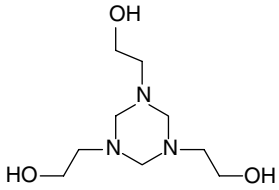
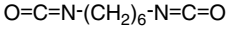
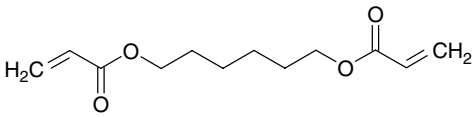
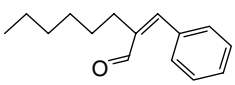
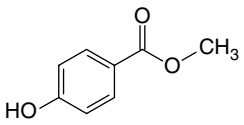
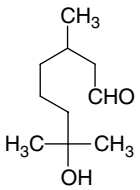
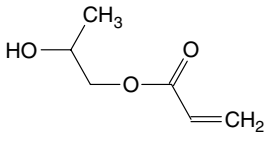
Appendix B (Continued)

Substance/Structure	CAS No.	Use			Human Data			Animal Data	
		Consumer Use	Industrial Use	Other Use	Clinical	Cross Reaction	Experimental	Positive	Negative
Butynediol 	110-65-6	-/-	x		x			x	x
Chlorhexidine and its salts[b] 	55-56-1	h/c		x	x			x	x
4-Chloro-*m*-cresol 	59-50-7	h/-	x	x	x	x		x	x
Cinnamyl alcohol[a] 	104-54-1	h/c		x	x		p/n	x	x
Citronellal[a] 	106-23-0	-/c		x	x	x	p/n	x	x
Citronellol[a] 	106-22-9	-/c		x	x	x	n		x
Coumarin[a] 	91-64-5	-/c		x	x		n	x	x
o-Cresyl glycidyl ether 	26447-14-3	-/-	x		x	x		x	
Diaminoazobenzene 	538-41-0	-/c	x		x	x		n.d.	
4,4′-Diaminodiphenylamine 	537-65-5	-/c	x		x	x		x	x

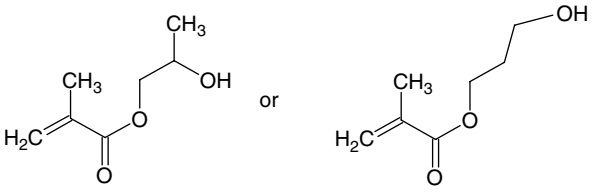
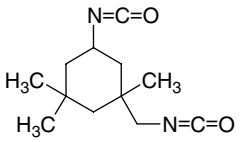
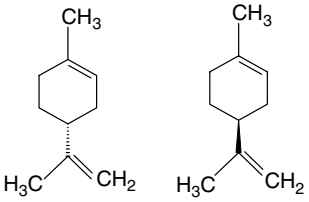
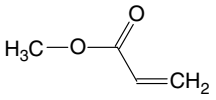
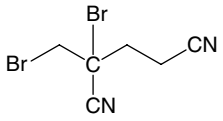
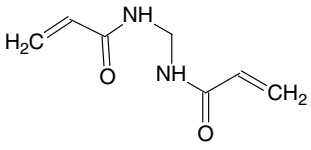
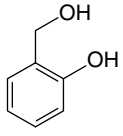
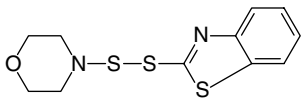
Appendix B (Continued)

Substance/Structure	CAS No.	Use			Human Data			Animal Data	
		Consumer Use	Industrial Use	Other Use	Clinical	Cross Reaction	Experimental	Positive	Negative
2,5-Dichlorobenzene sulfonic acid methyl ester 	78150-04-6	-/-		x	x	x		x	
Dicyclohexylmethane diisocyanate 	5124-30-1	-/-	x		x			x	x
Diethylene glycol dimethacrylate 	2358-84-1	-/c	x		x	x		x	x
Diethylthiourea[b] 	105-55-5	-/-	x	x	x	x		x	
N,*N*-Diisopropylbenzothiazyl-sulfenamide 	95-29-4	-/-	x		x	x		n.d.	
N-Dimethylbutyl-*N*′-phenyl-*p*-phenylenediamine 	793-24-8	-/-	x		x	x	n	x	x
Diphenylmethane diisocyanate 	101-68-8	-/-	x	x	x			x	
Disperse Blue 106 	12223-01-7	h/-	x		x	x		x	
Disperse Blue 124 	61951-51-7	h/-	x		x	x		x	
Dithiobisbenzo thiazole 	120-78-5	-/-	x		x	x	n	x	
Ethyl acrylate 	140-88-5	-/-	x		x		p	x	x

Appendix B (Continued)

Substance/Structure	CAS No.	Use			Human Data			Animal Data	
		Consumer Use	Industrial Use	Other Use	Clinical	Cross Reaction	Experimental	Positive	Negative
2-Ethylhexyl acrylate 	103-11-7	h/-	x	x	x	x		x	
Geraniol[a] 	106-24-1	h/c			x		p/n	x	x
Grotan BK 	4719-04-4	-/-	x		x			x	
1,6-Hexamethylene diisocyanate 	822-06-0	-/-	x	x	x			x	
Hexanediol diacrylate 	13048-33-4	-/-		x	x			x	x
Hexyl cinnamaldehyde 	101-86-0	-/c			x		n	x	x
Hydroxybenzoic acid methyl ester[b] 	99-76-3	-/c		x	x	x	n	x	x
Hydroxycitronellal 	107-75-5	h/c			x		n	x	
2-Hydroxypropyl acrylate[b] 	999-61-1	-/-		x	x			x	x

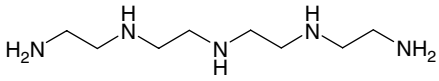
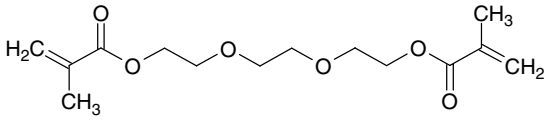
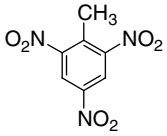
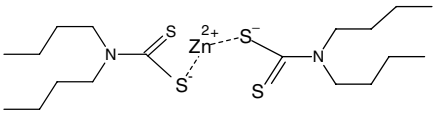
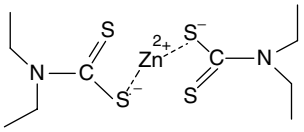
Appendix B (Continued)

Substance/Structure	CAS No.	Use			Human Data			Animal Data	
		Consumer Use	Industrial Use	Other Use	Clinical	Cross Reaction	Experimental	Positive	Negative
Hydroxypropyl methacrylate[b] 	923-26-2	h/c		x	x	x		x	x
Isophorone diisocyanate 	4098-71-9	-/-	x		x	x		x	
Limonene[a] 	138-86-3	h/-	x		x		n	x	x
Methyl acrylate 	96-33-3	-/-		x	x			x	x
Methyldibromo glutaronitrile 	35691-65-7	h/c	x		x		n	x	
Methylene-*bis*-acrylamide 	110-26-9	-/-	x	x	x	x			n.d.
2-Methylol phenol[b] 	90-01-7	-/-	x	x	x	x		x	
Morpholinyl-dithiobenzothiazol 	95-32-9	-/-	x		x	x			n.d.
Palladium chloride	7647-10-1	-/-	x		x			x	

Appendix B (Continued)

Substance/Structure	CAS No.	Use			Human Data			Animal Data	
		Consumer Use	Industrial Use	Other Use	Clinical	Cross Reaction	Experimental	Positive	Negative
Phenyl-α-naphthylamine	90-30-2	-/-	x		x	x		x	
o-Phenylenediamine	95-54-5	-/-	x		x	x		x	x
Phenyl isothiocyanate	103-72-0	-/-	x		x			x	
Phosphorus sesquisulfide	1314-85-8	h/-			x			n.d.	
Phthalic anhydride	85-44-9	-/-	x		x			x	
α-Pinene and its oxidation products[a]	80-56-8	-/c	x		x		p	x	
β-Pinene[a]	127-91-3	-/-	x		x			n.d.	
Piperazine[b]	110-85-0	-/-	x	x	x	x			x
Resorcinol[b]	108-46-3	-/c	x	x	x	x	n	x	x
2′,3,4′,5-Tetrabromo salicylanilide[b]	4372-73-0	-/-		x	x			x	x
Tetraethylene glycol dimethacrylate	109-17-1	-/-	x		x			x	x

Appendix B (Continued)

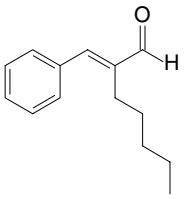
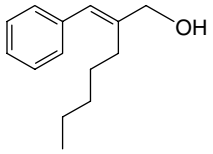
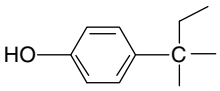
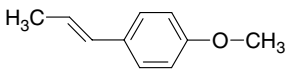
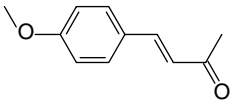
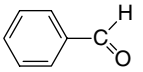
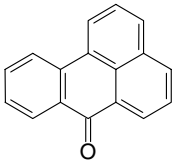
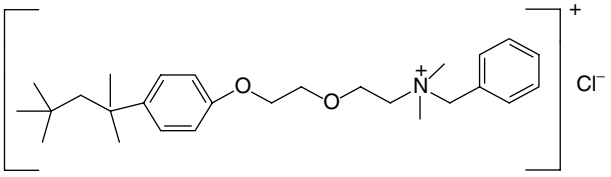
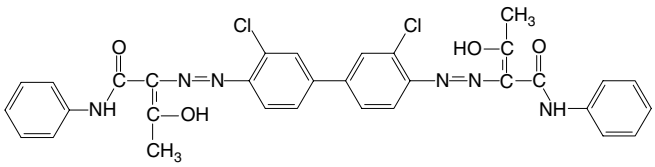
Substance/Structure	CAS No.	Use			Human Data			Animal Data	
		Consumer Use	Industrial Use	Other Use	Clinical	Cross Reaction	Experimental	Positive	Negative
Tetraethylene pentamine 	112-57-2	-/-	x		x	x		x	
Toluene diisocyanate $C_9H_4N_2O_2$	26471-62-5	-/-	x		x			x	x
Triethylene glycol dimethacrylate 	109-16-0	-/c	x	x	x	x		x	x
Trinitrotoluene 	118-96-7	-/-	x		x			x	
Zinc dibutyldithiocarbamate[b] 	136-23-2	-/-	x	x	x			x	
Zinc diethyldithiocarbamate 	14324-55-1	-/-	x	x	x	x		x	x

Note: h: household; c: cosmetics; p: positive; n: negative; n.d.: no data.

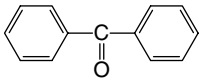
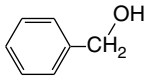
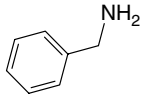
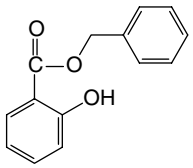
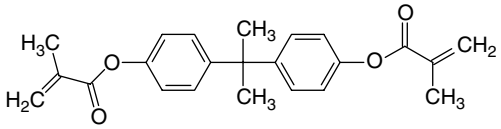
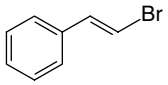
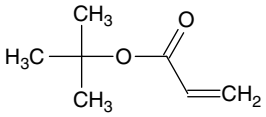
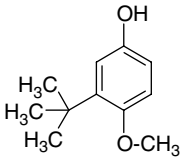
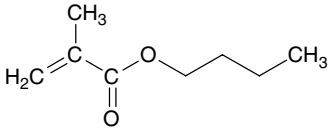
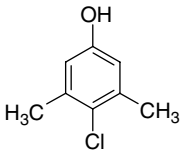
[a] Natural constituent of plants.

[b] Other use, i.e., as dental fillings, flavoring substances, substances used for research and development, pharmaceuticals, and ingredients of pharmaceuticals.

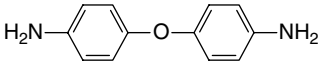
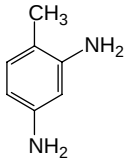
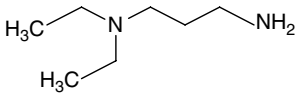
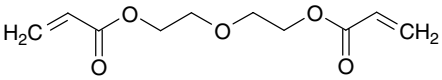
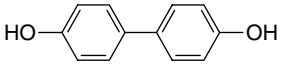
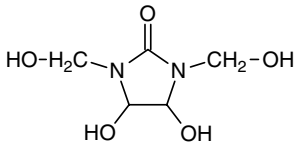
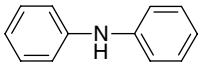
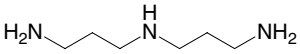
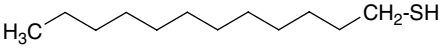
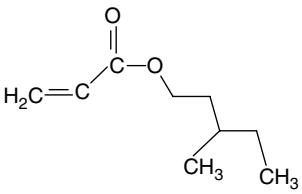
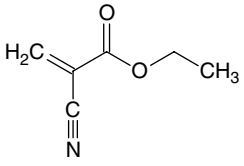
Appendix C: Summary of Data on Substances Listed in Category C

Substance/Structure	CAS No.	Use			Human Data			Animal Data	
		Consumer Use	Industrial Use	Other Use	Clinical	Cross Reaction	Experimental	Positive	Negative
α-Amyl cinnamaldehyde[a,b] 	122-40-7	-/c		x	x		n		x
α-Amyl cinnamyl alcohol[b] 	101-85-9	-/c		x	x		n		x
p-tert-Amylphenol 	80-46-6	-/-	x		n. d.			x	
Anethole[a,b] 	104-46-1	h/c		x	x		n	x	x
Anisylidene acetone 	943-88-4	-/c			x		p	n.d.	
Benzaldehyde[a] 	100-52-7	-/c	x	x	x		n	x	x
Benzanthrone 	82-05-3	-/-	x		x			x	
Benzethonium chloride[b] 	121-54-0	-/c		x	x	x		n.d.	
Benzidine Yellow 	6358-85-6	-/c		x	x			n.d.	

Appendix C (Continued)

Substance/Structure	CAS No.	Use			Human Data			Animal Data	
		Consumer Use	Industrial Use	Other Use	Clinical	Cross Reaction	Experimental	Positive	Negative
Benzophenone 	119-61-9	-/c	x		x	x	n		x
Benzyl alcohol[b] 	100-51-6	-/c	x	x	x		n	x	x
Benzylamine 	100-46-9	-/-	x		x			n.d.	
Benzyl salicylate[a] 	118-58-1	-/c			x		n	x	x
Bisphenol A dimethacrylate[b] 	3253-39-2	-/-	x	x	x (?)			x	
Bromostyrene 	103-64-0	-/c					n	x	x
tert-Butyl acrylate 	1663-39-4	-/-		x	x			x	
Butyl hydroxyanisole[b] 	25013-16-5	-/c		x	x			n.d.	
n-Butyl methacrylate[b] 	97-88-1	-/c	x	x	x	x		x	x
Chloroxylenol[b] 	88-04-0	-/c	x	x	x		p/n	n.d.	
Citronella oil[a]	8000-29-1	h/c			x		n	x	x

Appendix C (Continued)

Substance/Structure		CAS No.	Use			Human Data			Animal Data	
			Consumer Use	Industrial Use	Other Use	Clinical	Cross Reaction	Experimental	Positive	Negative
4,4′-Diamino diphenyl ether		101-80-4	-/-	x		n.d.	x		x	
2,4-Diaminotoluene		95-80-7	-/c	x		x			x	x
N,*N*-Diethylamino propylamine		104-78-9	-/-	x		x			x	
Diethyleneglycol diacrylate		4074-88-8	-/-	x		x			n.d.	
Dihydroxy diphenyl[b]		92-88-6	-/-	x	x	x			n.d.	
Dimethylol dihydroxy ethylene urea		1854-26-8	-/-	x	x	x			n.d.	
Diphenylamine[b]		122-39-4	-/c	x	x	x		n	x	
Dipropylene triamine		56-18-8	-/-	x		n.d.			x	x
Dodecyl mercaptan		112-55-0	-/-	x	x	x	x		x	
2-Ethylbutyl acrylate		3953-10-4	-/-		x	x			n.d.	
Ethyl cyanoacrylate		7085-85-0	h/c	x	x	x			n.d.	

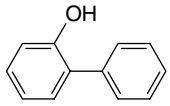
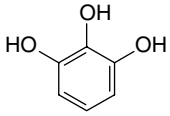
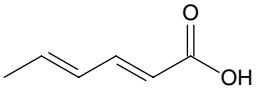
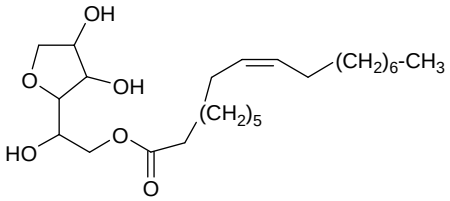
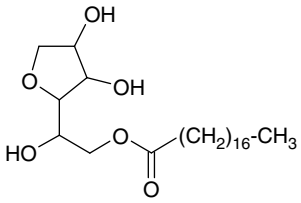
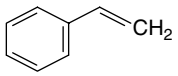
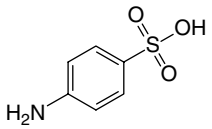
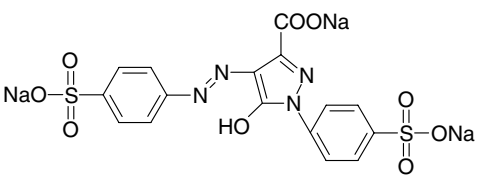
Appendix C (Continued)

Substance/Structure	CAS No.	Use			Human Data			Animal Data	
		Consumer Use	Industrial Use	Other Use	Clinical	Cross Reaction	Experimental	Positive	Negative
Ethylenediaminetetraacetic acid and its alkali salts	60-00-4	h/c	x	x	x			x	x
n-Hexyl acrylate	2499-95-8	-/-		x	n.d.	x		x	
Malachite Green	569-64-2	-/-	x		x				x
Maleic anhydride	108-31-6	-/-	x		x			x	x
Methyl salicylate[a,b]	119-36-8	-/c	x	x	x		n	x	x
Methyl vinyl ketone	78-94-4	-/-	x		x			x	
β-Naphthol[b]	135-19-3	-/c	x	x	x			x	
1-Naphthylamine	134-32-7	-/-	x		x			x	
Neopentanediol diacrylate	2223-82-7	-/-		x	n.d.			x	
Neopentyl acrylate	4513-36-4	-/-		x	n. d.			x	

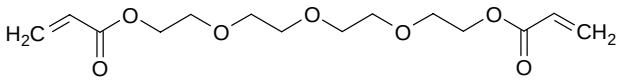
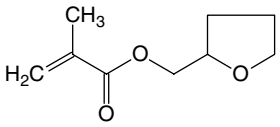
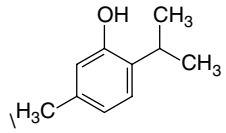
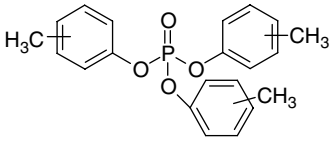
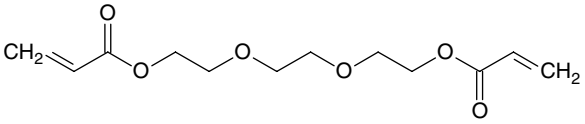
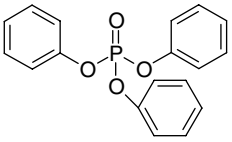
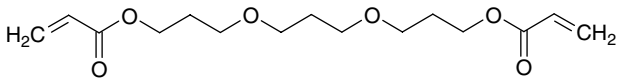
Appendix C (Continued)

Substance/Structure	CAS No.	Use			Human Data			Animal Data	
		Consumer Use	Industrial Use	Other Use	Clinical	Cross Reaction	Experimental	Positive	Negative
Neopentyl methacrylate	2397-76-4	-/-		x	n.d.			x	
Nigrosine	8005-03-6	-/-	x	x	x				x
1,5-Pentanediol dimethacrylate	13675-34-8	-/-		x	n.d.			x	
n-Pentyl acrylate	2998-23-4	-/-		x	n.d.			x	
p-Phenetidine	156-43-4	-/-	x		x			x	
2-Phenoxyethanol[b]	122-99-6	-/c	x	x	x			x	x
Phenyl acetaldehyde[a]	122-78-1	-/-	x	x	x	x	p	x	
m-Phenylenediamine	108-45-2	-/c	x		x			x	x
Phenylhydroxylamine	100-65-2	-/-		x	x		p	x	
Phenylmethyl chlorophenol	120-32-1	-/-		x	x		n	x	x

Appendix C (Continued)

Substance/Structure		CAS No.	Use			Human Data			Animal Data	
			Consumer Use	Industrial Use	Other Use	Clinical	Cross Reaction	Experimental	Positive	Negative
o-Phenylphenol[b]		90-43-7	-/-	x	x	x		n		x
Polyethylene glycol[b]	$H(OCH_2CH_2)_nOH$ ($n = 4 - 115000$)	25322-68-3	-/c	x	x	x				x
Propylene glycol[b]	$CH_3\text{-}CHOH\text{-}CH_2OH$	57-55-6	-/c	x	x	x		n		x
Pyrogallol[b]		87-66-1	-/c		x	x			x	x
Sorbic acid[a,b]		110-44-1	-/c	x	x	x		p/n	x	x
Sorbitan monooleate[b]		1338-43-8	-/c		x	x			n.d.	
Sorbitan monostearate[b]		1338-41-6	-/c		x	x			n.d.	
Sorbitan sesquioleate[b]		8007-43-0	-/c		x	x			n.d.	
Styrene		100-42-5	-/-	x		x			x	x
Sulfanilic acid		121-57-3	-/-	x		n.d.			x	x
Tartrazine[b]		1934-21-0	-/-		x	x			x	

Appendix C (Continued)

Substance/Structure	CAS No.	Use			Human Data			Animal Data	
		Consumer Use	Industrial Use	Other Use	Clinical	Cross Reaction	Experimental	Positive	Negative
Tetraethylene glycol diacrylate 	17831-71-9	-/-	x		x				x
Tetrahydrofurfuryl methacrylate 	2455-24-5	-/-	x		x				x
Thymol[a] 	89-83-8	-/c	x		x				x
Tricresyl phosphate 	1330-78-5	-/-	x		x		p/n		x
Triethylene glycol diacrylate 	1680-21-3	-/-	x		x		n		x
Triphenyl phosphate 	115-86-6	-/-	x		x			n.d.	
Tripropylene glycol diacrylate 	68901-05-3	-/-		x	x			x	x

Note: h: household; c: cosmetics; p: positive; n: negative; n.d.: no data.

[a] Natural constituent of plants.

[b] Other use, i.e., as dental fillings, flavoring substances, substances used for research and development, pharmaceuticals, and ingredients of pharmaceuticals.

92 Use of Modified Forearm Controlled Application Test to Evaluate Skin Irritation of Lotion Formulations

Miranda A. Farage

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92.1 INTRODUCTION

Skin effects of paper products are a combination of the inherent chemical irritation of the materials that make up the product and some degree of mechanical irritation due to friction during use. Safety evaluation programs on paper products usually involve test methods designed to detect potential irritation under exaggerated conditions. Typically, the evaluation begins with patch testing to confirm an absence of frank irritation, followed by a simulated use test. Often, a clinical study will be conducted as a final step in the evaluation process.

Modern facial tissue products are mild to skin. In fact, the current emphasis of manufacturers is to develop products that will provide skin benefits, rather than just an absence of negative effects. For example, a number of products are currently marketed that contain a lotion coating to aid in the healing or prevention of irritation that may occur with repeated, frequent use of the tissue product by cold or allergy sufferers.

A number of short-term screening test protocols have been developed to evaluate the potential benefits of lotion or moisturizer products with regard to the effects on normal skin, the ability to accelerate healing in irritated skin, and the prevention of irritation. Many of these test systems use the flexor surface of the forearm as a test site. Serup [1] evaluated intact skin using a single application of moisturizers to the flexor surface of the forearm in the absence of any pretreatment to induce irritation. The responses of the treated skin were assessed by measurements of epidermal hydration, scale pattern, and skin surface lipids. In a study conducted by Jemec and Na [2], volunteers used moisturizers on the volar surface of the forearm for 21 days: once a day on one arm and twice a day on the other arm. The mechanical properties of the skin were measured, as was skin capacitance.

To evaluate effects on irritated skin, a number of protocols have used skin irritated by pretreatment with sodium lauryl sulfate (SLS), which is widely used as a model irritant in studies on skin effects [3]. Blanken et al. [4] used a protocol that interspersed 45 min applications of a low concentration of SLS (0.5%) with applications of fatty preparations over a period of 2 weeks, and evaluated the efficacy of the preparations using measures of skin vapor loss. De Paepe et al. [5] developed a model to support efficacy claims of body lotions. The model consisted of applying body lotion formulations to the skin of the flexor surface of the forearm twice a day for 2 weeks to normal skin and skin that had been pretreated via a 24-h patch with 1.25% SLS. Responses were evaluated

Farage MA, Ebrahimpour A, Steimle, B. Englehart J, Smith DS. Evaluation of lotion-coated tissues using the modified forearm controlled application test method. *Skin Research and Technology* 13; 268–279, 2007. Reproduced with permission from Blackwell Publishing.

using transepidermal water loss (TEWL) and capacitance measurements.

Held et al. [6] used a 24-h patch pretreatment with 0.5% SLS to induce mild irritation on the upper arms and forearms to compare the efficacy of several different moisturizer formulations. These investigators used a similar model to evaluate the effects of moisturizers on the susceptibility to irritants [7]. Test subjects applied moisturizers to the upper arms and forearms three times daily for 5 days. This was followed by a 24-h patch of 0.25% SLS. Reactions were evaluated by a variety of methods, including visual scoring for erythema and TEWL. Zhai et al. [8] used a single pretreatment of test sites on the flexor aspect of the forearms with a dimethicone protectant lotion or vehicle control. This pretreament was followed by a 24 h occluded patch with 0.5% SLS. The effectiveness of the protectant lotion was determined by visual scoring, TEWL, skin color (a^* value), and cutaneous blood flow volume.

In cold sufferers, the irritation around the nostrils is a combination of effects, including the mucous running from the nose, the inherent irritant properties of the tissue components (chemical irritation), and mechanical irritation from friction resulting from frequent and repeated rubbing of the skin site with the tissue. Adding lotion to the tissue adds another source of potential irritating materials that may further contribute to the overall irritation, especially under conditions of application to damaged skin. We were interested in developing a model that incorporated all the elements that contribute to the complicated conditions experienced by an individual suffering from cold or allergies, such as chemical irritation of the tissue and lotion components, and mechanical irritation, in order to evaluate the potential skin benefits of various lotion formulations. We chose to adapt a test system that has been used to evaluate personal cleansing products and baby wipes, and is a modification of the forearm controlled application technique (FCAT) [9–11].

The objective of these studies was to evaluate the efficacy of different lotion formulations on facial tissues in preventing irritation, or aiding in the healing of irritation. We added a patch test with SLS to the modified FCAT protocol, to simulate an underlying irritation such as that which may exist in a cold sufferer. Test sites were treated by repeated wiping with facial tissues to simulate the product use by a cold sufferer. Visual assessment of erythema and dryness was used to determine if skin benefit(s) were detected.

92.2 METHODOLOGY

92.2.1 Test Protocols

The basic test protocol and protocol variations are presented in Figure 92.1. Within this basic design, several modifications were used, including variations in the concentration of SLS used, the number of total tissue wipes, and the number of days of grading. Specific details of the protocol are given in the appropriate figure or table legend.

In each experiment, two to three test sites were identified and demarcated on each volar surface of the forearm. Test sites measured 4 cm × 4 cm, and were 4 cm apart. Each site was treated with a different test product. The products were randomized, and the technician conducting the test was not aware of the test product identity.

For the tissue wipes, each tissue was folded up to five times, and wiped 10 times in a back and forth movement (20 passes). The test tissue was then refolded and the wiping repeated with a fresh area of the tissue. New tissues were used, as needed until the total number of back and forth wipes was completed. The SLS was patched using a Webril® patch (Professional Medical Products Company) covered by an occlusive, hypoallergenic tape (Blenderm®, 3M Company).

Basic protocol

Day 0		Day 1			Day 2	Day 3	Day 4
Grade	Patch	Grade	Tissue wipe	Grade	Grade	Grade	Grade
Baseline	24-h SLS	Postirritation		Post–tissue wipe	24-h post–tissue wipe	48-h post–tissue wipe	72-h post–tissue wipe

Variation 1

Day 0	Day 1				Day 2	Day 3
	Grade	Tissue wipe	Grade	Patch	Grade	Grade
	Baseline		Post–tissue wipe	24-h SLS	24-h postirriation	48-h post irritation

Variation 2

Day 0		Day 1			Day 2			Day 3	Day 4
Grade	Patch	Grade	Tissue wipe	Grade	Grade	Short tissue wipe	Grade	Grade	Grade
Baseline	24-h SLS	Postirritation		Post–tissue wipe			Post–2nd tissue wipe	48-h post–2nd tissue wipe	72-h post–2nd tissue wipe

FIGURE 92.1 Basic protocol design and variations. Grading is via visual scoring for erythema and dryness. TEWL was recorded for one experiment. (Adapted from Farage, M.A., Ebrahimpour, A., Steimle, B., Englehart, J., and Smith, D., Evaluation of the modified forearm controlled application test to evaluate skin irritation of lotion formulations, *Skin Research and Technology*, 2007, 13, 268–279. With permission from Blackwell Publishing.)

Grading of the test sites was done at intervals shown in Figure 92.1. When SLS patching was conducted, the patches were removed 30–60 min before grading. In all studies, visual scoring was conducted by expert graders under a 100 W incandescent daylight bulb. Erythema and dryness were graded on separate scales, according to a previously described scale of 0–6. On both scales, "0" indicated perfect skin and "6" indicated severe erythema and severe scaling with bleeding cracks for erythema and dryness, respectively [11]. Evaluation of irritation reactions by individuals specifically trained to assess skin reactions, i.e., expert graders, has been used for decades in a variety of protocol designs [9,12–15]. In an earlier publication, a direct comparison of visual and instrumental scoring methods was made [11]. The results of the visual scoring method were very similar to those of the instrumental scoring methods.

In some studies, reactions were also evaluated using measurement of TEWL (DermaLab® Evaporimeter Cortex Technology, Denmark). Prior to this measurement, subjects remained in a humidity- and temperature-controlled room for approximately 20 min to equilibrate with the ambient conditions.

92.2.2 Materials Tested

Samples evaluated in the program were either currently or recently marketed facial tissue products (with or without lotion coating), or the currently marketed facial tissue substrate coated with various test lotions. The components of the various lotion formulations tested are given in Table 92.1. All lotions were extruded onto the same tissue substrate at a concentration of 5 lb of lotion per 3000 ft^2 (or 0.815 mg/cm^2) of tissue substrate unless otherwise indicated.

92.2.3 Test Subjects

The protocol was approved by the test facility's Institutional Review Board. Participants in all studies were healthy female adult volunteers, aged 18–55 years, who had signed an informed consent. Subjects were excluded from participation if (1) they were currently participating in any other clinical study; (2) they had participated in any type of research study involving the forearms within the previous 21 days; (3) they had allergies to soap, detergent, perfume, cosmetics, or toiletries; (4) they were taking anti-inflammatory corticosteriods or other medications that may interfere with test results; (5) they had had eczema or psoriasis within the past 6 months; (6) they had been diagnosed with skin cancer within the previous 12 months; (7) they were pregnant or lactating; or (8) they had cuts, scratches, rashes, or any condition on their inner forearms that may prevent a clear assessment of their skin.

Eligible subjects were given a sensitive skin care cleansing bar for all bathing, and showering needs to be used beginning with their enrollment into the study and until their participation in the study was complete. They were instructed to avoid scrubbing the inner forearm areas and allow the soap and water to flow over the areas without washing. In addition, they were required to refrain from using lotions, creams, ointments, oils, or moisturizers on the forearm areas. Study groups consisted of 13–19 subjects.

92.2.4 Analyses of Data

The mean score for erythema (±standard error [SE]) for the panel was determined at each scoring time point. The postwipe average was calculated using the average of all

TABLE 92.1
Ingredients in Lotion Formulations Tested

	Currently Marketed Lotions			Experimental Lotions			
Lotion Code	**P-CSA**	**Q-SA[a]**	**B-SA**	**B-CSA**	**A-SA**	**A-CSA**	**C**
Formula base	Mineral oil	Mineral oil and isopropyl palmitate	Mineral oil and petrolatum	Mineral oil and petrolatum	Petrolatum	Petrolatum	—
Fatty alcohol	Cetearyl alcohol	Stearyl alcohol	Stearyl alcohol	Cetearyl alcohol	Stearyl alcohol	Cetearyl alcohol	—
Other components	Paraffin wax, steareth-2, aloe, and vitamin E	Cerasin, calendula oil, dimethicone	—	—	Fatty acid sucrose esters	Fatty acid sucrose esters	Fatty acid sucrose esters
Lotion concentration on tissue substrate	0.815 mg/cm^2 (unless otherwise stated)	Unknown	0.815 mg/cm^2	0.815 mg/cm^2	0.815 mg/cm^2	0.815 mg/cm^2	0.815 mg/cm^2
Silicone version	Lotion was extruded onto tissue substrate with either 3000 or 4500 ppm silicone	A version with silicone is also available	—	—	Lotion was extruded onto tissue substrate with 3000 ppm silicone	—	—

[a] Q-SA is a currently marketed competitor's product. Some of the product details are unknown.

Source: Farage, M.A., Ebrahimpour, A., Steimle, B., Englehart, J., and Smith, D., Evaluation of the modified forearm controlled application test to evaluate skin irritation of lotion formulations, *Skin Research and Technology*, 2007, 13, 268–279. With permission from Blackwell Publishing.

postwipe visit scores for each subject, then calculating the mean (±SE) over all subjects. If there were missing visits for a subject, that subject was not included in the calculation of the postwipe average.

In those experiments where SLS patching occurred prior to treatment with the lotion, the results are presented as the change in group mean. The change in group mean was calculated by determining the change from post-SLS baseline (i.e., the postirritation score) for each subject, then calculating the average over all subjects. In some cases, not all test subjects completed the entire test. In these instances, the scores recorded for the dropped subjects were removed from the calculation of the change in group mean for that time point.

All statistical analyses are described in the table and figure legends.

92.3 RESULTS

92.3.1 Establishing the Basic Protocol

The model was examined by assessing the reactions in the presence and absence of pretreatment by patching with SLS, followed by wiping with several lotion formulations. Typical results are shown in Table 92.2. Using lotion P-CSA in the absence of pretreatment with SLS, there is virtually no evidence of dryness or erythema resulting from wiping with a lotion-coated tissue. However, patching with SLS establishes an underlying irritation as shown by an increase in both dryness and erythema.

TABLE 92.2
Effect of P-CSA Lotion in Preventing SLS-Induced Irritation

Scoring Time Point	*n*	Dryness (Mean ± SE)	Erythema (Mean ± SE)
Tissues with P-CSA lotion (no SLS pretreatment)			
Baseline	18	0.00 ± 0.00	0.00 ± 0.00
Post–tissue wipe	18	0.00 ± 0.00	0.11[a] ± 0.05
24 h post–tissue wipe	18	0.11 ± 0.08	0.00 ± 0.00
48 h post–tissue wipe	18	0.06 ± 0.06	0.00 ± 0.00
72 h post–tissue wipe	18	0.11 ± 0.08	0.00 ± 0.00
Tissues with P-CSA lotion (pretreatment with SLS)			
Baseline	16	NA	0.00 ± 0.00
Postirritation	16	1.62 ± 0.24	1.09 ± 0.11
Post–tissue wipe	16	0.31[b] ± 0.15	1.44[b] ± 0.13
24 h post–tissue wipe	16	0.44[b] ± 0.13	1.12 ± 0.15
48 h post–tissue wipe	16	0.19[b] ± 0.10	0.91 ± 0.15
72 h post–tissue wipe	16	0.19[b] ± 0.10	0.59[b] ± 0.13

Note: The basic test protocol was used, as shown in Figure 92.1. Test sites on the flexor surfaces of the forearms were pretreated by 24 h patch with 0.1% SLS. After patch removal, test sites were wiped with the test tissues using a total of 120 wipes (240 passes). NA = not available.

[a] Significantly different from baseline score ($p < .05$).

[b] Significantly different from postirritation score ($p < .05$).

Source: Farage, M.A., Ebrahimpour, A., Steimle, B., Englehart, J., and Smith, D., Evaluation of the modified forearm controlled application test to evaluate skin irritation of lotion formulations, *Skin Research and Technology*, 2007 13, 268–279. With permission from Blackwell Publishing.

92.3.2 Effect of Lotion on Prevention of Irritation

Sites were treated by wiping with the tissues prior to patching with SLS (protocol variation 1 in Figure 92.1) to evaluate the effectiveness of two different lotion formulations in preventing irritation. Results of the scoring for dryness and erythema are shown in Figure 92.2. For all four treatments, scoring immediately after the tissue wipes (post–tissue wipe grade) showed no significant increase in either dryness or erythema compared to baseline values. Scores at both time points after patching with SLS (24 and 48-h postirritation) were significantly elevated for all four treatments. However, there were no significant differences between the reactions at sites treated with the lotioned products and their nonlotioned controls, although the dryness scores tended to be lower when the tissue contained lotion.

92.3.3 Effect of Lotion on Healing Irritation

We examined the effect of various lotion products on the speed of recovery of skin irritated by patching with SLS. The same lotion formulations evaluated in the previous experiment were evaluated in this protocol, using the basic protocol (as shown in Figure 92.1). Results from lotion P-CSA are shown in Table 92.3. The presence of P-CSA lotion on the tissues had a significant effect on dryness. Immediately following the tissue wipe, the lotioned tissue caused a substantial reduction in dryness from a mean group score of 1.06–0.39: a change in group mean score of –0.67. The nonlotioned tissue produced a much more modest reduction in dryness, with a change in the group mean score of –0.18. The mean scores for dryness at the lotion-treated sites were significantly lower than the control sites at two of the three time points. The erythema reactions for both the lotioned and nonlotioned tissues increased slightly immediately following the tissue wiping procedure, and remained elevated for the duration of the test. Determining the average change in dryness and erythema for all post–tissue wipe scores (i.e., post–tissue wipe average) provides a means to rank order test materials for overall benefits. In this instance, the test tissue with lotion P-CSA produced a greater reduction in average dryness scores than the test tissue without lotion (–0.48 versus –0.06). The average change in erythema scores was similar for the two test products (0.36 and 0.32).

Figure 92.3 presents the results for both test products (lotion P-CSA and lotion Q-SA) from this same experiment as the change in group mean scores (dryness and erythema) from those scores recorded after patching with 0.25% SLS. The presence of lotion P-CSA directionally reduced dryness on test sites pretreated by SLS patching, with the results reaching significance at two scoring time points (Figure 92.3a). The presence of lotion Q-SA did not produce significant improvements in dryness compared to the nonlotioned control. Lotion P-CSA produced directionally less erythema than lotion

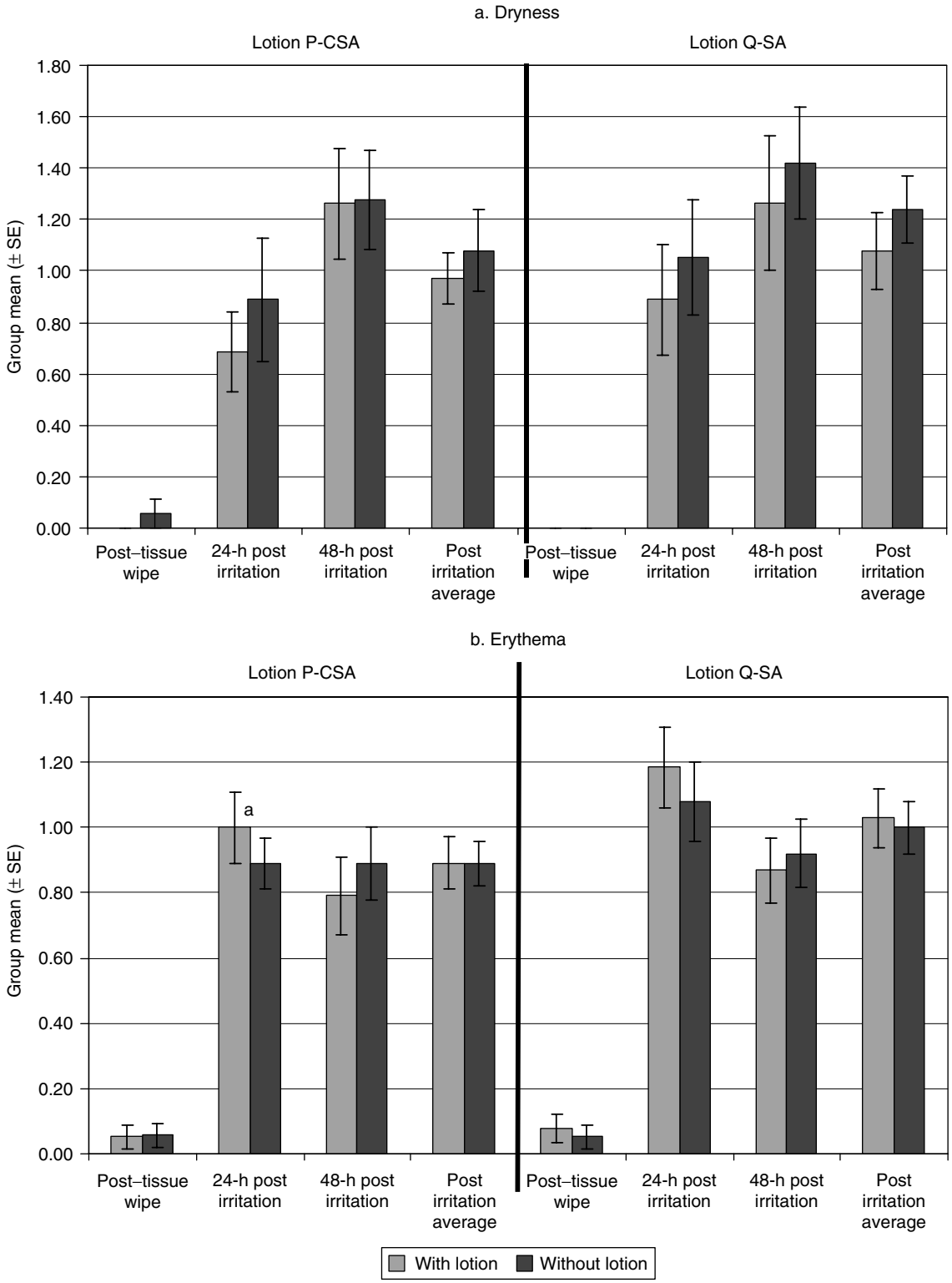


FIGURE 92.2 Effect of pretreatment with lotion. Protocol variation 1 was used, as shown in Figure 92.1. Test sites on the flexor surfaces of the forearms of 19 subjects were wiped with the test tissues on day 1 using a total of 200 wipes (400 passes). This was followed by a 24 h occlusive patch with 0.25% SLS. The group mean scores for dryness (a) and erythema (b) were determined for each scoring time point. Postirritation average treatment comparisons were performed using analysis of variance (ANOVA). All other treatment comparisons were performed using the stratified cochran-mantel-haentzel (CMH) test. For lotion P-CSA, the concentration of lotion on the tissues was 0.668 mg/cm^2. Since Q-SA is a currently marketed competitor's product, the lotion concentration is unknown. [a]Control for P-CSA (without lotion) different from Q-SA (with lotion) ($p < .05$). (Adapted from Farage, M.A., Ebrahimpour, A., Steimle, B., Englehart, J., and Smith, D., Evaluation of the modified forearm controlled application test to evaluate skin irritation of lotion formulations, *Skin Research and Technology*, 2007, 13, 268–279. With permission from Blackwell Publishing.)

TABLE 92.3
Effects of Pretreatment with P-CSA Lotion on Healing SLS-Induced Irritation

		Dryness		Erythema	
Scoring Time Point	*N*	Group Mean Score (Mean ± SE)	Change from Postirritation Score (Mean ± SE)	Group Mean Score (Mean ± SE)	Change from Postirritation Score (Mean ± SE)
Tissues with P-CSA lotion					
Baseline	18	0.06 ± 0.06	—	0.00 ± 0.00	—
Postirritation	18	1.06 ± 0.19	—	0.92 ± 0.10	—
Post–tissue wipe	18	0.39 ± 0.12[a]	–0.67 ± 0.21	1.25 ± 0.13	0.33 ± 0.08
24 h post–tissue wipe	18	0.89 ± 0.11	–0.17 ± 0.22	1.33 ± 0.17	0.42 ± 0.16
48 h post–tissue wipe	18	0.44 ± 0.12[a]	–0.61 ± 0.26	1.25 ± 0.22	0.33 ± 0.21
Post–tissue wipe average	18	0.57 ± 0.08[a]	–0.48 ± 0.22	1.28 ± 0.14	0.36 ± 0.12
Control (tissues with no lotion)					
Baseline	17	0.11 ± 0.08	—	0.00 ± 0.00	—
Postirritation	17	0.94 ± 0.10	—	0.85 ± 0.09	—
Post–tissue wipe	17	0.76 ± 0.11	–0.18 ± 0.15	1.15 ± 0.12	0.29 ± 0.06
24 h post–tissue wipe	17	1.12 ± 0.12	0.18 ± 0.18	1.29 ± 0.08	0.44 ± 0.12
48 h post–tissue wipe	17	0.76 ± 0.11	–0.18 ± 0.15	1.09 ± 0.18	0.24 ± 0.17
Post–tissue wipe average	17	0.88 ± 0.06	–0.06 ± 0.13	1.18 ± 0.10	0.32 ± 0.09

Note: The basic test protocol was used, as shown in Figure 92.1, except scoring was not conducted at 72 h post–tissue wipe. Test sites on the flexor surfaces of the forearms were pretreated by 24 h patch with 0.25% SLS. After patch removal, test sites were wiped with the test tissues (tissues with 0.668 mg/cm^2 lotion P-CSA) using a total of 200 wipes (400 passes). The change from the postirritation score was determined for each subject, and then the average over all subjects was calculated. The post–tissue wipe average was calculated using the average of all postwipe scores for each subject, then calculating the average over all subjects. Treatment comparisons for erythema for post–tissue wipe time points was performed using ANCOVA. Post–tissue wipe average treatment comparisons were performed using ANOVA. All other comparisons were performed using stratified CMH.

[a] Significantly lower than control ($p < .05$).

Source: Farage, M.A., Ebrahimpour, A., Steimle, B., Englehart, J., and Smith, D., Evaluation of the modified forearm controlled application test to evaluate skin irritation of lotion formulations, *Skin Research and Technology*, 2007, 13, 268–279. With permission from Blackwell Publishing.

Q-SA, although the results did not reach significance (Figure 92.3b). As indicated by the postwipe average scores, the test tissue with P-CSA lotion produced the greatest average improvement in dryness scores of the four test products. The test tissue with Q-SA produced the highest average level of erythema.

92.3.4 Evaluation of Formula Options

The effects of two lotion formulations with and without silicone on dryness and erythema were compared (Figure 92.4). One of these formulations (P-CSA) was a mineral oil base with cetearyl alcohol. The other (A-SA) was a petrolatum base with stearyl alcohol. In the absence of silicone, both formulas caused a reduction in dryness from the post–tissue wipe score (Figure 92.4a). In the case of lotion P-CSA, this reduction was substantial; however, it did not reach statistical significance. The addition of 3000 ppm silicone resulted in even more marked improvements in dryness scores for both lotion formulations. Erythema scores in the presence of silicone tended to be greater (Figure 92.4b). As indicated by the postwipe averages, lotion P-CSA with 3000 ppm silicone produced the greatest overall reduction in dryness. However, this formula combination also produced the greatest overall increase in erythema.

The effects of different levels of silicone in the presence of the same lotion formulation were also evaluated. Figure 92.5 shows a comparison between test products in which lotion P-CSA was applied to tissue substrate containing no silicone, 3000 ppm silicone, or 4500 ppm silicone. The presence of 3000 ppm silicone produced slightly greater reductions in dryness at some time points than the lotion in the absence of silicone (Figure 92.5a). Increasing the silicone to 4500 ppm reversed this trend, with lesser reductions in dryness. The levels of erythema were slightly improved in both silicone test groups compared to tissues with no silicone (Figure 92.5b). In this study, measures of TEWL were also included in the evaluation (Figure 92.5c). The TEWL results indicated slight improvements with either 3000 or 4500 ppm silicone compared to no silicone.

An experiment was conducted to compare the relative skin effects of cetearyl and stearyl alcohol. Two different lotion base formulations were tested: one with a petrolatum base and one with a mineral oil/petrolatum base. Each formulation was prepared using ceateryl alcohol and stearyl alcohol. All other components of the formulations were identical. As seen in Figure 92.6, the stearyl alcohol gave results very similar to those of the cetearyl alcohol for both dryness and erythema.

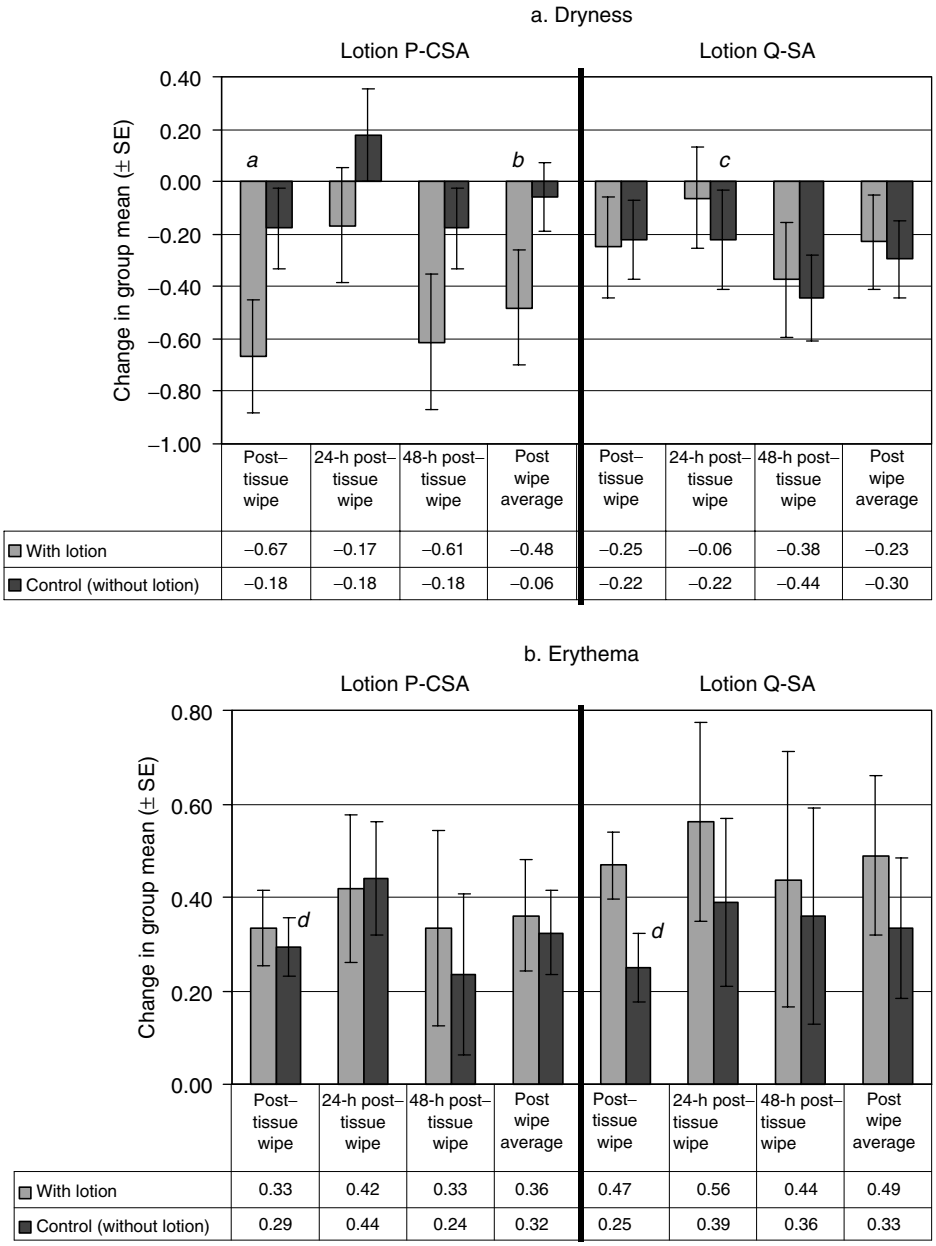
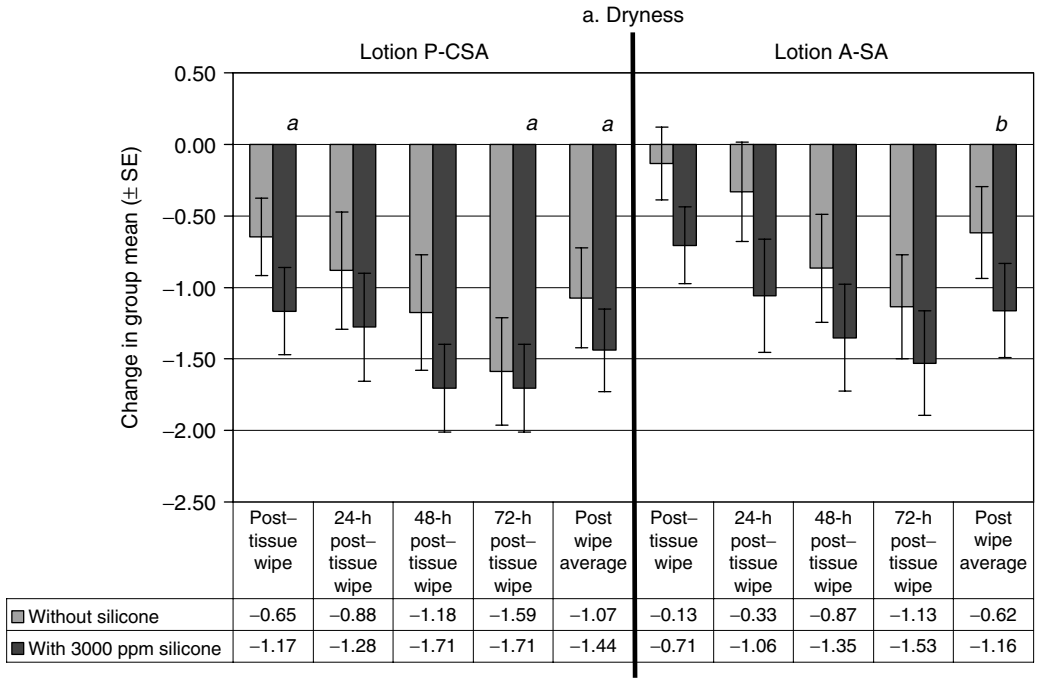
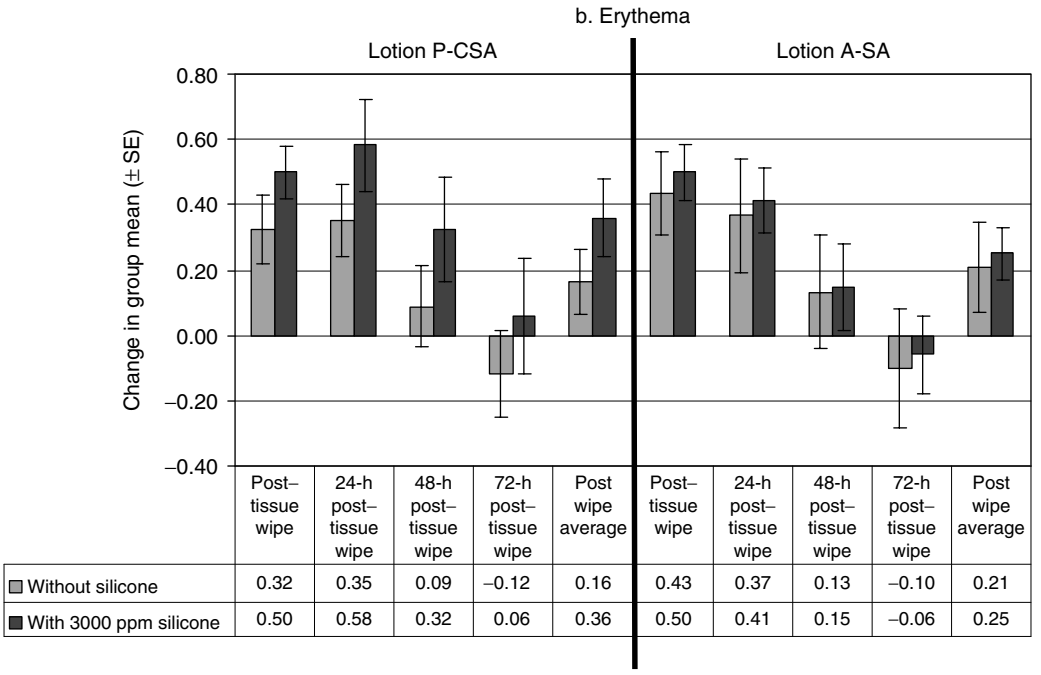


FIGURE 92.3 Effect of lotion treatment on SLS-patched skin. The basic test protocol was used, as shown in Figure 92.1. Experimental details are described in Table 92.3. The postwipe average was calculated using the average of all postwipe visits for each subject, then calculating the average over all subjects. The changes from the postwipe scores are given. Treatment comparisons for the change in erythema at 24 and 48 h post–tissue wipe, and the change in postwipe average were performed using ANOVA on ranks. All other treatment comparisons were performed using the stratified CMH. The concentration of P-CSA lotion on the tissues was 0.668 mg/cm^2. [(a)]P-CSA (with lotion) different from control Q-SA (without lotion) ($p < .05$). [(b)]P-CSA (with lotion) different from control P-CSA (without lotion) ($p < .05$). [(c)]Control Q-SA (without lotion) different from control P-CSA (without lotion) ($p < .05$). [(d)]Controls P-CSA and Q-SA (without lotion) different from Q-SA (with lotion) ($p < .05$). (Adapted from Farage, M.A., Ebrahimpour, A., Steimle, B., Englehart, J., and Smith, D., Evaluation of the modified forearm controlled application test to evaluate skin irritation of lotion formulations, *Skin Research and Technology*, 2007, 13, 268–279. With permission from Blackwell Publishing.)

Four formulations with different emollients were compared. These formulations consisted of a mineral oil base (P-CSA), a mineral oil/petrolatum base (B-CSA), a petrolatum base (A-CSA), and a proprietary mixture of fatty acid sucrose esters (C). Results (shown in Figure 92.7) indicated that all four formulations had comparable benefits with regard to the effects on dryness (Figure 92.7a). However, the test sites treated with proprietary formulation (C) exhibited less erythema than the other formulations at the final two scoring time points (48 and 72 h post–tissue wipe in Figure 92.7b). The change in the 48-h score was significantly different from the other three formulations. The change in the 72 h score was significantly different from formulation P-CSA.



	Post–tissue wipe	24-h post–tissue wipe	48-h post–tissue wipe	72-h post–tissue wipe	Post wipe average	Post–tissue wipe	24-h post–tissue wipe	48-h post–tissue wipe	72-h post–tissue wipe	Post wipe average
Without silicone	−0.65	−0.88	−1.18	−1.59	−1.07	−0.13	−0.33	−0.87	−1.13	−0.62
With 3000 ppm silicone	−1.17	−1.28	−1.71	−1.71	−1.44	−0.71	−1.06	−1.35	−1.53	−1.16

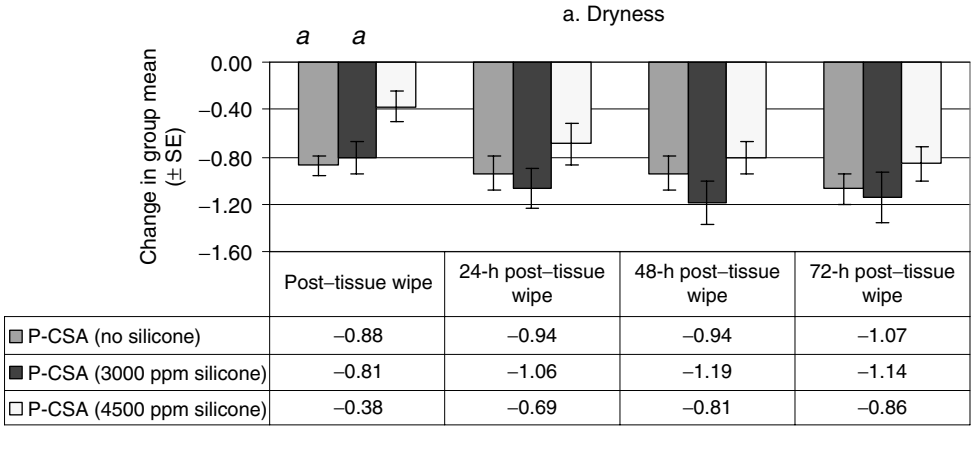


	Post–tissue wipe	24-h post–tissue wipe	48-h post–tissue wipe	72-h post–tissue wipe	Post wipe average	Post–tissue wipe	24-h post–tissue wipe	48-h post–tissue wipe	72-h post–tissue wipe	Post wipe average
Without silicone	0.32	0.35	0.09	−0.12	0.16	0.43	0.37	0.13	−0.10	0.21
With 3000 ppm silicone	0.50	0.58	0.32	0.06	0.36	0.50	0.41	0.15	−0.06	0.25

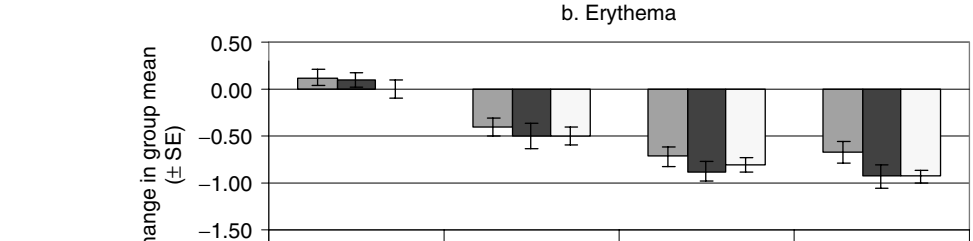
FIGURE 92.4 Effect of silicone on dryness and erythema. The basic test protocol was used, as shown in Figure 92.1, with the same experimental details as described in Figure 92.3 and Table 92.3. The postwipe average and the changes from the postwipe scores were determined as described above. Erythema post–tissue wipe average comparisons were performed using ANOVA. All other treatment comparisons were performed using ANOVA on ranks. The concentration of the lotions on the tissues was 0.668 mg/cm^2 for P-CSA and 0.815 mg/cm^2 for A-SA. For both formulations, the amount of silicone on the tissue substrate was 3000 ppm. [(a)]P-CSA (with silicone) different from A-SA (without silicone) ($p < .05$). [(b)]A-SA (with silicone) different from P-CSA (without silicone) ($p < .05$). (Adapted from Farage, M.A., Ebrahimpour, A., Steimle, B., Englehart, J., and Smith, D., Evaluation of the modified forearm controlled application test to evaluate skin irritation of lotion formulations, *Skin Research and Technology*, 2007, 13, 268–279. With permission from Blackwell Publishing.)

92.4 DISCUSSION

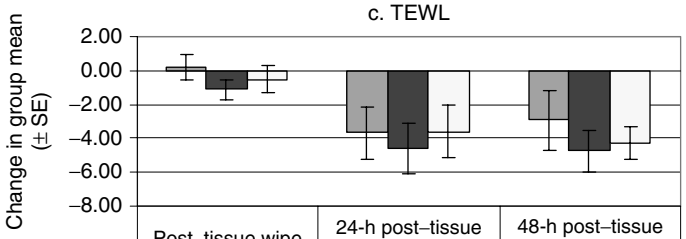
Consumers suffering from colds and allergies are particularly vulnerable to the irritant effects of repeated, frequent use of tissue products. These effects include the inherent chemical irritation of the tissue substrate and lotion materials, and the mechanical irritation from friction. We were interested in developing a model that included these elements as a way to



	Post–tissue wipe	24-h post–tissue wipe	48-h post–tissue wipe	72-h post–tissue wipe
P-CSA (no silicone)	–0.88	–0.94	–0.94	–1.07
P-CSA (3000 ppm silicone)	–0.81	–1.06	–1.19	–1.14
P-CSA (4500 ppm silicone)	–0.38	–0.69	–0.81	–0.86



	Post–tissue wipe	24-h post–tissue wipe	48-h post–tissue wipe	72-h post–tissue wipe
P-CSA (no silicone)	0.13	–0.41	–0.72	–0.68
P-CSA (3000 ppm silicone)	0.09	–0.50	–0.88	–0.93
P-CSA (4500 ppm silicone)	0.00	–0.50	–0.81	–0.93



	Post–tissue wipe	24-h post–tissue wipe	48-h post–tissue wipe
P-CSA (no silicone)	0.21	–3.68	–2.91
P-CSA (3000 ppm silicone)	–1.13	–4.58	–4.74
P-CSA (4500 ppm silicone)	–0.52	–3.61	–4.27

FIGURE 92.5 Effects of different levels of silicone. Protocol variation 2 was used, as shown in Figure 92.1. Test sites on the flexor surfaces of the forearms of 13–16 subjects were pretreated by 24 h patch with 0.1% SLS. After patch removal, test sites were wiped with the test tissues using a total of 120 wipes (240 passes). On the following day, the test sites were wiped with the test tissues for an additional 10 wipes (20 passes). The changes from the postwipe scores are shown. Comparisons for dryness and erythema were performed using stratified CMH. Comparisons for TEWL were performed using analysis of covariance (ANCOVA). [(a)]P-CSA (no and 3000 ppm silicone) different from P-CSA (4500 ppm silicone) ($p < .05$). (Adapted from Farage, M.A., Ebrahimpour, A., Steimle, B., Englehart, J., and Smith, D., Evaluation of the modified forearm controlled application test to evaluate skin irritation of lotion formulations, *Skin Research and Technology*, 2007, 13, 268–279, 2007. With permission from Blackwell Publishing.)

quickly screen candidate materials for lotion coatings, and as a means of claims support. A number of investigators have used applications of materials to the flexor surface of the forearm, with and without pretreatment with SLS, as a means of evaluating the effectiveness of lotions. We added an element to this approach by incorporating repeated wipes with the lotion-coated tissues to include a mechanical irritation component.

The results shown in Table 92.2 confirm that treatment with the lotion-coated tissue in the absence of SLS pretreament results in no visible irritation, as indicated by virtually no increase in either dryness or erythema scores. Therefore, pretreatment with SLS to establish a background level irritation was essential to enable the detection of differences in irritant effects.

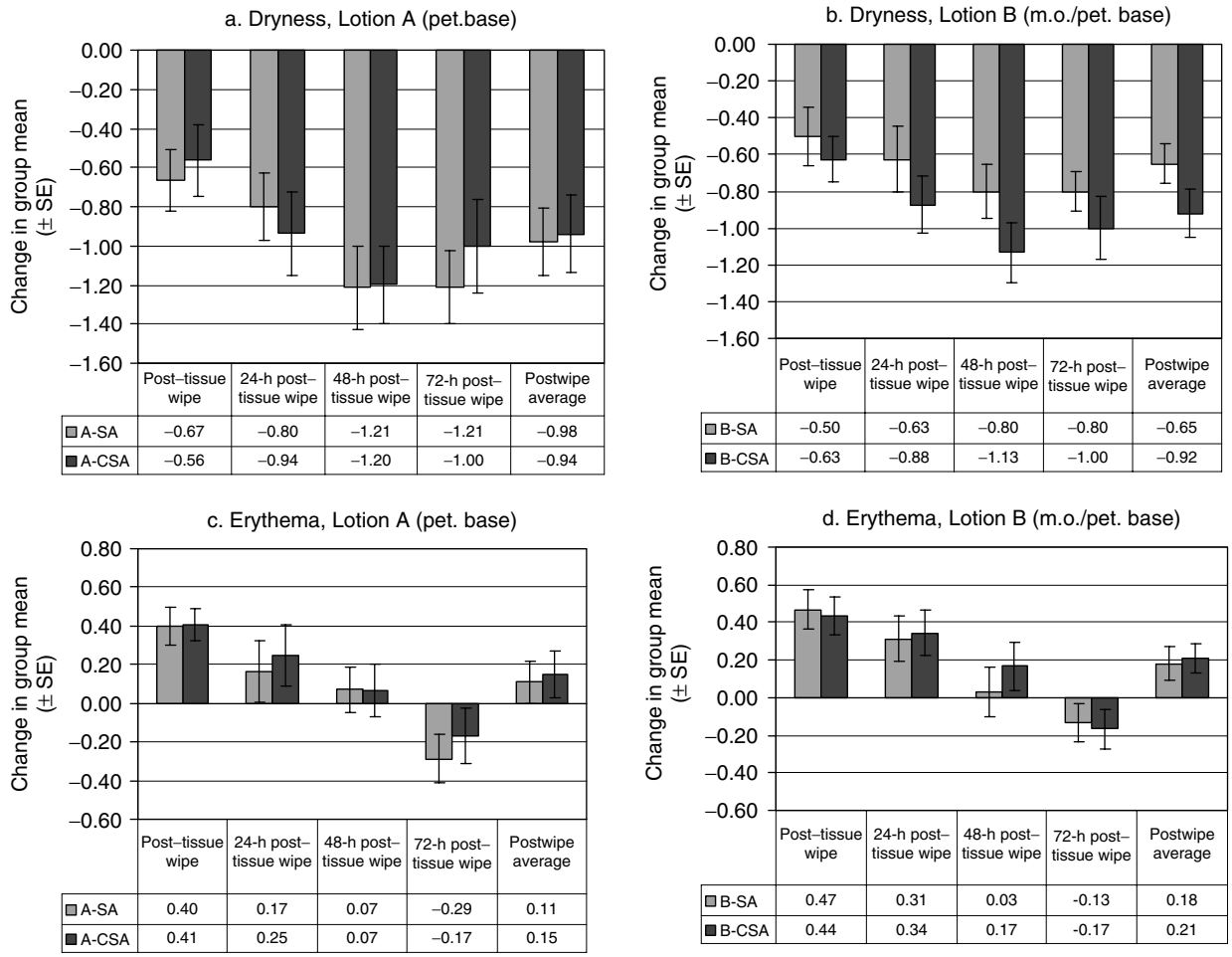


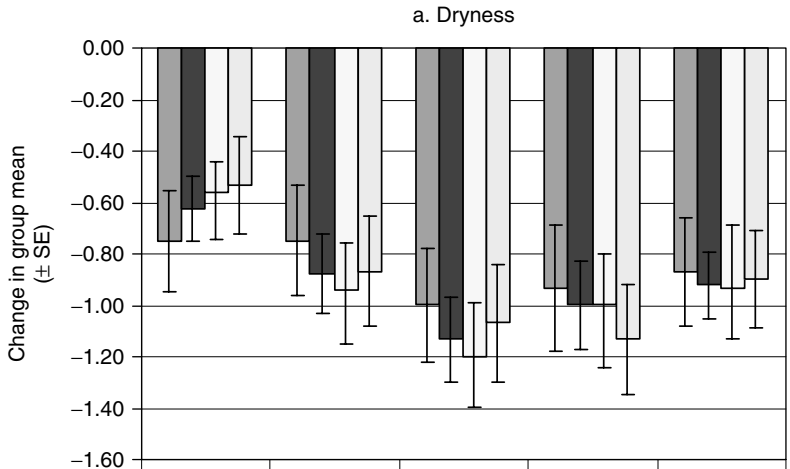
FIGURE 92.6 Comparison of stearyl and cetearyl alcohol in two different base formulations. The basic test protocol was used, as shown in Figure 92.1. Test sites on the flexor surfaces of the forearms of 15–16 subjects were pretreated by 24 h patch with 0.1% SLS. After patch removal, test sites were wiped with the test tissues using a total of 120 wipes (240 passes). Tissues were coated with either base formula A containing either stearyl or cetearyl alcohol (A-SA and A-CSA, respectively), or base formula B containing either stearyl or cetearyl alcohol (B-SA and B-CSA, respectively). The changes from the postwipe scores are shown. Comparisons were performed using ANOVA. Differences between treatments did not reach significance. (Adapted from Farage, M.A., Ebrahimpour, A., Steimle, B., Englehart, J., and Smith, D., Evaluation of the modified forearm controlled application test to evaluate skin irritation of lotion formulations, *Skin Research and Technology*, 2007, 13, 268–279. With permission from Blackwell Publishing.)

Two lotion formulations were tested for protective effects against irritation by SLS, P-CSA and Q-SA, as shown in Figure 92.2. As expected, such short-term treatments with lotion were not particularly effective in preventing irritation, although dryness scores tended to be slightly lower when lotion was present on the tissue. Interestingly, Zhai et al. [8] reported a significant reduction in both visual scores and TEWL when a single application of dimethicone was used prior to patching with 0.5% SLS. In our study, the competitive product (Q-SA) contained dimethicone; however, there was no indication of a protective effect.

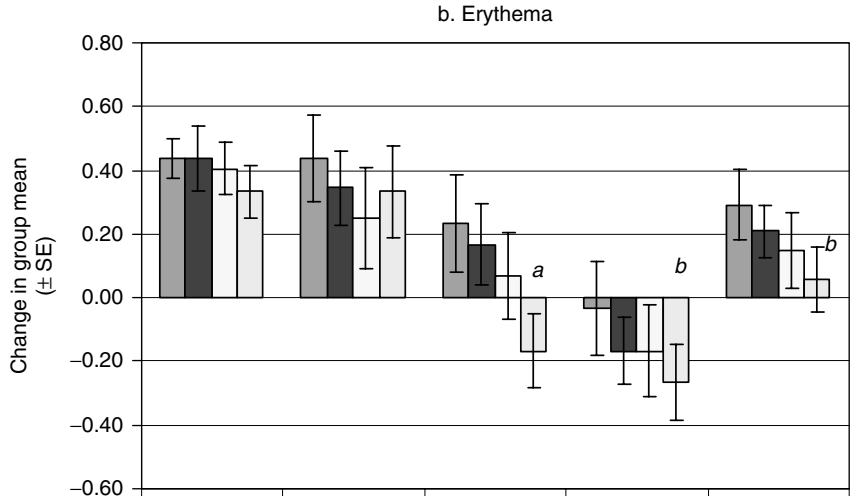
These same lotions were tested for healing effects after SLS pretreatment (Figure 92.3). Lotion P-CSA was effective in reducing dryness, compared to the tissue without lotion, but was not effective in treating erythema. Lotion Q-SA did not appear to have an effect in reducing dryness compared to the tissue without lotion, and the presence of the Q-SA lotion actually increased erythema (significantly, at post–tissue wipe scoring time point). Both of these lotion formulations contain mineral oil. Lotion Q-SA also contains isopropyl palmitate and dimethicone. The lotions also differ in the fatty alcohol, with P-CSA containing cetearyl alcohol and Q-SA containing stearyl alcohol.

In the study shown in Figure 92.4, the effectiveness of a silicone layer on the tissue in healing SLS-induced irritation was evaluated for two formulations: P-CSA (mineral oil base with cetearyl alcohol) and A-SA (petrolatum base with stearyl alcohol). For formula P-CSA, the presence of silicone improved the dryness scores, as shown by a greater reduction in scores at individual time points than the same lotion without silicone, and a greater postbaseline average reduction (Figure 92.4a). For lotion A-SA, silicone had a similar, although less pronounced, effect. However, the presence of silicone also increased the erythema scores slightly over lotion without silicone.

The improvement in dryness scores and increase in erythema scores when silicone was added to P-CSA lotion were



	Post–tissue wipe	24-h post–tissue wipe	48-h post–tissue wipe	72-h post–tissue wipe	Postwipe average
P-CSA	–0.75	–0.75	–1.00	–0.93	–0.87
B-CSA	–0.63	–0.88	–1.13	–1.00	–0.92
A-CSA	–0.56	–0.94	–1.20	–1.00	–0.93
C	–0.53	–0.87	–1.07	–1.13	–0.90



	Post–tissue wipe	24-h post–tissue wipe	48-h post–tissue wipe	72-h post–tissue wipe	Postwipe average
P-CSA	0.44	0.44	0.23	–0.03	0.29
B-CSA	0.44	0.34	0.17	–0.17	0.21
A-CSA	0.41	0.25	0.07	–0.17	0.15
C	0.33	0.33	–0.17	–0.27	0.06

FIGURE 92.7 Comparison of emollient bases. The test conditions were identical to those described for Figure 92.6. Comparisons were performed using ANOVA. [a]C different from P-CSA, B-CSA, and A-CSA ($p < .05$). [b]C different from P-CSA ($p < .05$). (Adapted from Farage, M.A., Ebrahimpour, A., Steimle, B., Englehart, J., and Smith, D., Evaluation of the modified forearm controlled application test to evaluate skin irritation of lotion formulations, *Skin Research and Technology*, 2007, 13, 268–279. With permission from Blackwell Publishing.)

not repeated in an experiment comparing different levels of silicone (Figure 92.5). In this experiment, the dryness scores without silicone and with 3000 ppm silicone were much more similar than the results shown in Figure 92.4. An increase in silicone to 4500 ppm appeared to dramatically reduce the improvement in dryness scores. There were differences in the study protocols that may have contributed to this finding. In the study shown in Figure 92.4, a greater number of total tissue wipes were used (200 versus 130), and a higher concentration of SLS was used in the pretreatment (0.25% versus 0.1%) compared to the study shown in Figure 92.5. This may account for some differences in the results. However, an evaluation of experiments in which the identical tissue and lotion combination was used, along with the two different concentrations of SLS, indicates that the scores resulting from pretreating with 0.25% SLS are similar in severity and the time of recovery

to the scores resulting from pretreating with 0.1% SLS (data not shown). Therefore, it is unlikely that the difference in the concentration of SLS used in pretreatment accounts for the different results in these two experiments.

Interestingly, the increase in erythema scores normally seen in all other studies was not observed in the results shown in Figure 92.5. After essentially no increase immediately after the tissue wipe, erythema dropped at each subsequent scoring time point. In all other studies, erythema tended to increase to a greater extent immediately after the tissue wipe, sometimes staying elevated for 24 or 48 h. It is not obvious why this should be the only study in which erythema scores decreased so consistently.

The relative effects of ceateryl and stearyl alcohol were compared in two different base formulations, as shown in Figure 92.6. These two alcohols had very similar effects in the two lotion formulations (lotion A which is a petrolatum base and lotion B which is a mineral oil/petrolatum base). The cetearyl alcohol showed slightly greater reductions in dryness than the stearyl alcohol in the mineral oil/petrolatum formula (lotion B) versus the petrolatum formula (lotion A) (Figures 92.6a,b). Both alcohols produced similar levels of erythema (Figures 92.6c,d).

Figure 92.7 shows the comparison of four formulations that differ in the base ingredients. Lotion P-CSA has a mineral oil base. Lotion B-CSA has a mixture of mineral oil and petrolatum. Lotion A-CSA has a petrolatum base. Lotion C is a proprietary mixture of fatty acid sucrose esters. All four formulations had comparable benefits with regard to dryness (Figure 92.7a). However, the test sites treated with proprietary formulation (C) exhibited significantly less erythema (Figure 92.7b) than the other formulations at the final two scoring time points (48 and 72 h post–tissue wipe), and significantly less overall (postbaseline average), indicating that this lotion formula base may provide greater skin benefits than the other mineral oil or petrolatum formulations.

The potential skin benefits from lotion-coated facial tissues are the result of a number of factors, including the condition of the skin, the type of tissue substrate, the composition of the lotion, the amount of lotion on the tissue, and the kinetics of the lotion transfer to the skin. Developing the best tissue product requires balancing the interactions of all these factors to find a combination of lotion and substrate that maximizes the skin benefits, while minimizing the potential for the chemical and mechanical irritation of damaged skin. Testing on normal skin will not adequately account for all of these factors. However, when the stratum corneum and its barrier properties were slightly damaged by pretreatment with SLS, materials from the lotion formulation can penetrate more easily, leading to an increase in chemical irritation and an ability to differentiate between products. This modification of the FCAT can be used to compare various lotion formulations for skin benefits and healing properties, and to qualitatively rank the benefits of various formulation options.

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93 Hair in Toxicology

Ken-ichiro O'goshi

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93.1 INTRODUCTION

Hair, scales, feathers, claws, horns, and nails are all derived from skin and so all consist of keratinized modified epidermal cells [1]. Hair and nails are of major interest to dermatologists, to toxicologists, and to those interested in forensic and medicolegal investigations. The slower growth of nails (toenail, 0.05 mm/day: finger nail, 0.1 mm/day) compared with human scalp hair fibers (around 0.35 mm/day), and the fact that nails (especially of the foot) are not normally exposed to external contaminants, make the nail particularly useful for retrospective analysis [2].

Morphological, serological, and chemical examination of human hair for medical purposes was initiated some decades ago. In the 1960s and 1970s, hair analysis was used to evaluate exposure to toxic heavy metals. At this time, examination of hair for organic substances, especially drugs, was hardly possible because analytical methods were not sensitive enough. Since the early 1980s, the development of highly sensitive and specific assay methods such as radioimmunoassay (RIA) or gas chromatography/mass spectrometry (GC/MS) has permitted the analysis of organic substances trapped in hair. This, theoretically, offered the possibility of revealing an individual's recent history of drug exposure beginning at sampling day and dating back over a period of weeks or months.

93.2 HAIR AND EXPOSURE TO ENVIRONMENTAL POLLUTANTS

93.2.1 Monitoring of Environmental Pollution

The environmental pollution in a number of industrial agglomerations continues to be high and so assessment of the degree of health risk involved is becoming a major public health concern in Europe, the United States, Canada, Japan, and several other developed countries and regions.

Underlying the growing interest among public health authorities in biomarkers of human exposure to environmental pollutants and the potential health risk related to this exposure is the difficulty to assess qualitatively and quantitatively the full extent of environmental pollution. Furthermore, analyses of air and surface water samples if collected nonsystematically yield virtually worthless data in this respect, as the degree of environmental contamination may be highly variable. For example, concentrations of air pollutants are influenced by actual weather conditions, by local air movements, or by the thermal inversions causing critical accumulation of emissions in given areas during smog episode. Moreover, the quality of surface water, especially in the rivers and streams, generally depends on flow rate that is the degree to which the incoming discharges are diluted by the stream flow. There may be fluctuation in the quality of surface water, influenced by the nature and amount of discharge of industrial effluents depending on the actual production technology used.

As an alternative way to the technical approach to this problem, biological indicators could be used to monitor pollution of the environment. This strategy would appear to be particularly well suited to demonstrate environmental pollution by potentially toxic trace elements, including trace metals [3–5].

For the assessment of human exposure, the examination of suitable human material would appear to be more appropriate than the analysis of plant or animal pollution. Human biological materials that are accessible for sampling usually include blood and urine. Successful attempts have also been made to measure the accumulation of metals, such as lead [6–8], and nonmetal pollutants, such as fluorine, in children. The nail and the hair are also becoming increasingly valuable as highly accessible tissues for the purpose of monitoring human exposure to environmental pollutants [8,9].

93.2.2 The Hair Fiber as a Biomarker of Human Exposure to Metals and Inorganic Substances

The hair fiber represents an easily accessible material for the noninvasive sampling of individuals or population groups, to assess criminal, occupational, or environmental exposure to toxic elements [10–17]. The current trend is toward the increasing use of the human hair fiber as the bioresource of choice for monitoring excessive exposure to trace elements. This development is closely linked with the availability of suitable analytical procedures, sensitive enough to quantify the content of the respective pollutant in the biological specimen tested.

In a historical way, there are several techniques of investigation to determine some elements of human hair such as the neuron activation (NAA) [18] for arsenic content, instrumental NAA (INAA) [19–22] for a wide spectrum of trace elements, atomic absorption spectrophotometry (AAS) for lead, cadmium, and nickel, X-ray fluorescence and proton-induced x-ray emission (PIXE) [17,23–25], and inductively coupled plasma mass spectrometry (ICP MS) [26]. Certified reference materials (CRM) are used to ensure accuracy of determination as a quality control (QC) standard.

The relationship between the arsenic content in hair and in blood determined by a destructive separation NAA technique in the early 1970s appeared to be the most appropriate method still relevant today. Urine and hair samples were analyzed using a classical colorimetric method [27]. To achieve sufficient accuracy using this test, not less than 1 g (usually about 2 g) of hair sample and 200 mL 24 h urine sample were

needed from each person for analysis. These data showed that the tests for arsenic content of hair, used for years to demonstrate arsenic exposure for the purpose of forensic or industrial toxicology [18,28,29], are equally applicable to environmental settings when groups are studied.

Hair samples are far simpler to collect, transport, and store than samples of blood and urine, and similarly their processing prior to analysis, provided that the content of potentially toxic elements is determined by adequate analytical techniques [17,30,31].

93.2.3 Advantages and Limitations of Hair Fiber Analysis as a Biomarker of Human Exposure to Trace Elements

Major advantages and limitations of the use of human hair analysis as a biomarker of excessive exposure to trace metals can be summed up as follows. The extent of human exposure to pollutants in the general environment does not as a rule reach the level of exposure in occupational settings, and varies greatly from individual to individual, thus leading to relatively great intragroup differences in values. The only rational approach that might help to overcome this problem is to use a representative group approach when assessing the risk of environmental exposure. There should be at least 20 individuals per population group to be sampled to ensure that the differences in element content of hair samples in these groups can be qualified, quantified, and analyzed statistically [32,33].

The accumulation of trace elements in hair might be, at least partially, dependent on age and sex. To overcome this problem, groups of 10-year-old boys have been used as the most suited representatives of nonoccupationally exposed populations under surveillance since the 1960s [34].

To date there have been no reliable data that would allow the establishment of generally applicable limits for normal content of individual trace elements in human hair. The elemental content of hair tends to vary from one geographical region to another, depending on natural background conditions, including composition of soil, element concentration in water and food, and eating habits [10–13,35]. Therefore, all findings obtained in the area under surveillance are compared to the values in suitable control groups of "unexposed" human populations.

Sampling, transport, and storage of hair samples are easier than the sampling, transportation, and processing of blood or urine samples, the most common specimens used today to demonstrate human exposure to a variety of noxious environmental agents [4,36,37]. Parts of human hair samples are also very easy to preserve for later control analysis.

93.2.4 Washing of Hair Samples

In contrast to blood and urine samples, hair should be cleared of external contaminants prior to elemental analysis. There are several different removal techniques in the past literatures, and the question of washing methods has received a great deal of attention. Because the metallic cations released into the washing solution might bind chemically with sulfur in the keratin matrix of the hair [38–40], it is obvious that a nonpolar solvent should be used as a first-step wash to remove, together with hair surface grease, the attached dust particles containing metals in undissolved form.

Excessive external contamination of hair usually reflects massive environmental pollution and resulting human exposure [41].

93.3 HAIR AND NUTRIENT/DIET ASSESSMENT

The appearance of an individual's hair has been known to reflect the diet and nutritional status. With the advent of sophisticated scientific analyses, we have realized that diet and nutritional information can also be gleaned from the chemical and elemental compositions of hair.

Hair can be a source of dietary and nutritional information at several levels, namely at the physical and structural levels, from incorporated atoms and molecules, and from chemical signals within constituent proteins, primarily the stable isotopic ratios of the light elements such as carbon, nitrogen, and sulfur. Oxygen and hydrogen isotopic distributions reflect environmental temperature and water sources indicative of an individual's place of origin. Atoms and molecules incorporated in the hair include organic metabolites and trace elements, particularly metals. Most organic metabolites that are detectable in hair are related to ingestion of substances other than food, mainly drugs and alcohols. Metals detectable in hair can be considered to be either nutritionally necessary, innocent contaminants, or toxic, and can be related to both dietary and nondietary exposures. Nutritionally desirable metals in hair are primarily derived from food [41]. Ingestion of toxic metals can be nondietary, for example, occupational exposure or accidental ingestion, and dietary sources via consumption of foods containing the metal, for example, mercury in fish. The elemental concentration of incorporated metals is usually measured for nutritional assessment. Isotropic ratios of metals, however, can also be measured. This approach provides information about an individual's place of origin, rather than about nutrition because the relative amounts of radiogenic isotopes, for example, strontium, lead, and rubidium, relate to the local geological environment.

93.3.1 Diet and Nutritional Investigations Using Hair

The assessment of dietary intake and the assessment of nutritional status are two very different issues and it is essential to distinguish between them. Diet can be defined as that which is intentionally consumed by an individual for optimal metabolic function. It is possible for individuals to consume widely differing diets, and yet be equal or similar in their nutritional status. Light stable isotope analysis of hair has been used to identify aspects of an individual's long-term diet type such as the importance of animal products, vegetables, and fish. This work has had most application in anthropology and links closely to the field of archeological science known

as paleodiet. A combination of dietary and environmental indicators has been used in forensic science for identification of an individual's place of origin, again tying in with archeological studies of human ecology and immigration.

The nutritional status of an individual or a population has been assessed by measurement of the nutritionally necessary elements including zinc, copper, manganese, chromium, selenium, calcium, sodium, and magnesium among others. This work is related to investigations of occupational/nonoccupational exposure to toxic metals including cadmium, lead, mercury, arsenic, aluminum, and chromium.

93.3.2 Advantage of Using Hair as a Study Tissue

The advantages of dietary or nutritional analysis of an individual using a hair sample are manifold. Hair is an ideal biopsy material, since it is available from almost all individuals, and is easy and painless to sample. From the point of physical and chemical stability, no special storage between sampling and analysis is required, unlike blood or urine. And its external growth in a linear fashion provides a longitudinal measure of body tissue synthesis and therefore potentially of variation in diet or nutrition over a long period of time.

Rapid developments in the sophistication of analytical techniques, together with relatively high concentrations of elements such as metals and nonmetals within hair mean that most analyses can be performed on samples of 1–2 mg. Hair proteins contain carbon, nitrogen, oxygen, hydrogen, and sulfur at percentages similar to or higher than those for other tissue proteins, and the hair shaft contains most of the trace metals at relatively high concentrations compared to the rest of the body [42,43].

In addition to noninvasive tissue sampling from living individuals, the tissue preserves well postmortem, making it a good forensic biopsy material. It has been more widely used in this context as a source of deoxyribo nucleic acid (DNA) evidence, but can also be used as a source of information on diet and geographical origin.

93.3.3 Problems Associated with the Use of Hair as a Study Tissue

Unfortunately, despite the inherent advantages of hair as a tissue for analysis, the problems associated with it are also many and widely documented. Trace metal analysis for nutritional assessment in particular is now widely discredited within the medical profession [44–49].

The associated problems vary, depending on the type of analysis being performed. Although the analysis of hair proteins is relatively trouble free, great difficulties are associated with regard to trace metal analysis of hair. These problems are mainly described as below:

- The association between the chemical signal measured and an individual's diet or nutritional status, namely the correlation issue relating to diet and body pools
- Endogenous variability in the chemical signal being measured
- Hair growth rate and its effect on the measured chemical signal
- Hair contamination

93.3.4 Correlation with Diet and Body Pools

The assumption underlying hair analysis for dietary or nutritional assessment is either that signals measured in hair can be correlated with diet, or that the signals in hair are representatives of those in other body tissues and the "metabolic pool." These are two significantly different conceptions, and problems of hair trace element analysis are often explained by unclear conceptions.

The relationship between trace metal concentrations in hair and that of the diet is not a simple one, and studies of the link between the two have produced conflicting results. It is important to recognize that trace metal concentration in the body is not solely dependent on the metal concentration in the diet but also dependent on other dietary constituents [50]. For instance, dietary phytate and fiber levels are known to affect zinc absorption [51]. Ascorbic acid increases the intake of inorganic iron but reduces copper absorption; competitive absorption in the gut between iron and manganese and between copper and zinc will affect the body levels of these pairs of elements [41,52,53]. Results of analyses of trace element levels in hair relative to the individual's nutritional status have consequently produced less than clear-cut results.

For nutritional assessment in a clinical setting, blood concentrations are generally considered as representatives of the "metabolic pool," and therefore, for hair to act as a proxy diagnostic tissue, hair concentrations must ideally correlate with blood concentrations. This has not been conclusively demonstrated with several studies reporting a lack of a significant correlation between hair and blood (serum and plasma) for a number of elements including zinc, copper, iron, calcium, and aluminum [45–62]. However, the exception is selenium that appears to be highly correlated in blood (serum) and hair [63].

93.3.5 Endogenous Variability in the Chemical Signal from Hair

Although diet is the primary surface of carbon and nitrogen in tissue, secondary influences including environmental and physiological factors have been reported to affect isotopic values. For nitrogen, the "trophic level enrichment" (i.e., the increase in nitrogen isotopic values between diet and body tissues [64]) between diet and hair is quite variable under a range of environmental and climatic conditions such as temperature, altitude, aridity as well as being affected by physiological factors such as water stress, starvation and growth, and diet macronutrient composition [65–74].

Hair color and location on the body appear to have no discernable effect on isotopic values. That is to say no difference

was found between the carbon and nitrogen isotopic values, i.e., the C/N ratio. Similarly, C/N ratios of pigmented and nonpigmented (gray) hairs were the same as isotopic values of hairs on different locations on the body [75,76].

Other than that due to diet, physiology, or environment, there appears to be little intrinsic variability in the carbon, nitrogen, sulfur, oxygen, or hydrogen isotopic variations within an individual's keratin protein [75,77].

Hair trace element concentrations have been reported to vary widely due to factors such as sex, age, race, hair color, and anatomic location of hair sampled. These parameters can cause as much variation in concentrations as can diet. The data are sometimes conflicting, and a conclusive relationship has seldom been established. As for studies investigating hair trace metal correlation with diet and other body pools, there is generally no consistency in sample collection methods and analytical techniques. Most worryingly, the sampling strategy is often *ad hoc*, and the majority of studies have, surprisingly, not even addressed for variation in dietary intake of food and water.

Differences in trace element concentrations between hair from males and females have been reported, yet again there is little consistency [78–83]. There are changes in hair metal concentrations with age, with significant variations observed during early infancy, puberty, pregnancy, and lactation, but yet again no clear patterns emerge [84,85,62,81–83,86]. However, pregnancy and oral contraceptive pills are well documented to alter hair copper levels [56,57]. Within the same individual, manganese levels have been reported to be higher in black hair than in white, while zinc and copper levels were reported to be similar [87].

Differences in calcium, manganese, strontium, iron, copper, selenium, and nickel were found between Caucasian, Negroid, and Asiatic samples; however, some of this difference may simply be due to differences in hair color or indeed due to differences in diet [80,88]. Anatomic variation across the body may also be a factor in variable metal concentrations.

93.3.6 Hair Growth Rate

Hair grows at a rate of 1 cm/month [89], but there are variations in the hair types, anatomic locations on the body, and hormonal levels. Where analytical techniques are independent of sample size, the rate of hair growth is largely irrelevant. The rate of hair growth can be used as a means of calibrating the longitudinal record of diet, and small variations due to season and other physiological factors will merely cause a slight error in the time period calculated. However, the analytical technique is not independent of sample size, if hair growth rates are substantially altered. This has major implications for interpretation of data. When diet is adequately supplied with macro- and micronutrients, hair growth will be unaffected by diet, but in severe cases of malnutrition or specific micronutrient deficiencies, particularly zinc, growth will be substantially slowed [90,91].

93.3.7 Hair Contamination

As an externally growing tissue, hair is subject to contamination, either endogenous, including sweat and sebaceous secretions, or exogenous from atmospheric dust and cosmetic treatments (shampoos, coloring, etc.), exacerbated by hair's hygroscopic nature [92].

The further the hair is from the scalp, the older it is, and the greater exposure time it will have to contaminants. Such exposure primarily may affect metal concentrations rather than changes in hair protein. Correlations between trace element concentration and distance from the scalp have been reported [80,86,93–95].

93.3.8 Analysis and Data Interpretation

Although there are still controversies on how to interpret the results, particularly concerning external contamination, cosmetic treatments, ethnical bias, or drug incorporation, pure analytical work in hair analysis has reached a sort of plateau, having solved almost all the analytical problems.

Trace metal analysis is more prone to difficulties. It is because metals can be lodged or adsorbed within the hair structure, but metals are themselves not an intrinsic component of them. Thus, without spatial resolution, it is impossible to distinguish between endogenous and exogenous materials.

93.4 HAIR AND METAL TOXICITY

Hair can be noninvasively obtained, easily stored and transported, and later analyzed for the presence of certain metals [96–98]. Hair analysis is most informative when the metal of interest is a xenobiotic and the exposure is major [99]. A large body of epidemiological evidence correlates hair mercury concentrations to blood mercury levels, and both to fish consumption in a dose–response fashion [99]. In addition, hair is one of several biomarkers used in epidemiological studies of arsenicosis and arsenic-contaminated drinking water. Further, hair analysis can be used clinically and in forensic medicine to document thallium poisoning, an intoxication that also results in pathological changes in the hair (Table 93.1).

Hair's utility as a biomarker is considered to be limited in most occupational environments where exposures are airborne. Hair is also subject to exogenous contamination by the toxicant of interest. Therefore, distinguishing metal content internally distributed and excreted into the hair after absorption from metals that externally contaminate the hair surface is difficult, if not impossible [97,99]. Likewise, hair has limited usefulness when the metal of interest is both a potential occupational exposure and an essential dietary trace element. In these cases, the metal may naturally be present in hair in varying amounts.

Timing is also important. Because of nonvascularity of the hair shaft and its growth rate, hair metal content is unlikely to accurately reflect very recent exposures for hours or several days. In addition, hair is also unlikely to document exposures that have occurred more than 1 year before the

TABLE 93.1
Metal Toxicity: Source of Exposure, Absorption Route, Target Organ of Toxicity, Half-Life in a Whole Body, Excretion, and Other Biomarkers but Hair

Metal	Source of Exposure	Absorption Route	Target Organ of Toxicity	Half-Life (Whole Body)	Excretion	Other Biomarkers but Hair
Mercury	Dental amalgams, thermometers, gas meters, fish, thiomerosal in vaccines	Inhalation of vapor (Hg0)	Kidney, CNS, PNS	60 days	Urine, feces	Urine
		Ingestion of seafood (Methyl Hg)	CNS	40–70 days	Feces	Blood and hair
		Parental form vaccines (Ethyl Hg)	CNS, kidney	7–20 days	Feces	Blood
Arsenic	Fish, shellfish, groundwater, (discrete geographic areas), byproducts of mining, smelting and coal-burning, pesticides	Ingestion of seafood, chemicals	Liver, kidney, CNS	3–5 days	Urine	Blood, urine
Lead	Leaded gasoline, lead paint in older houses	Inhalation of vapor	Blood, kidney reproductive, cardiovascular, CNS, PNS	Weeks (in blood), months (in soft tissues), 5–15 years (in bones)	Urine, feces, hair, nails, sweat	Blood, bone
Cadmium	Tobacco-smoking, electroplating, cadmium alloy, battery production, welding solders, cadmium pigment	Inhalation of vapor	Liver, kidney, CNS	10–30 years (in kidney), 5–10 years (in liver)	Urine	Blood, urine
Manganese	Manganese alloys, dry cell batteries, paints, fertilizers, gasoline additive (MMT), mining, smelting	Inhalation of vapor, gastrointestinal absorption	Liver, kidney, brain	10 months	Urine, feces, hair, nails, sweat	Blood, urine
Thallium	Rodenticide, manufacturing of special glass	Inhalation of vapor, gastrointestinal absorption, dermal absorption	No internal anatomic reservoir	2–30 days	Urine, feces, saliva, hair, nails	Blood, urine, hair, nails

Note: CNS, central nervous system; PNS, peripheral nervous system; MMT, methylcyclopentadienyl manganese tricarbonyl.

time of analysis [99]. In individuals with hair of sufficient length, however, segmental hair analysis may provide information regarding exposure over long time [99–101].

93.4.1 Mercury

93.4.1.1 Toxicology

Mercury is a xenobiotic used in chloralkali production, some electrical switches, fluorescent light bulbs, and certain batteries. Elemental mercury (dental amalgams, thermometers, and gas meters), methyl mercury (fish), and ethylmercury (thiomerosal in vaccines) are the forms and potential exposures most relevant to the general population [102,103]. For most people, background mercury exposure and variability in blood mercury result primarily from the consumption of fish containing methylmercury, which bioaccumulates in seafood worldwide [102–105].

Acute exposure to elemental mercury can produce acute lung injury, while chronic exposures may produce renal dysfunction and neuropathy. Frank poisoning results in additional neurological disturbances including tremor, behavioral changes such as erethism, gingivitis [106,107], and progression to delirium/hallucinations [108].

Clinical methylmercury intoxication is characterized by ataxia, tremor, constriction of visual fields, and cortical and cerebral atrophy [107,108] and is generally associated with hair levels greater than 50 ppm [109]. There is considerable debate regarding the "safe" dose of methylmercury from fish in the diet, particularly for children and woman of childbearing age [105,110,111]. Thimerosal or thiomersal contains ethylmercury, used as a vaccine preservative since the 1930s. Ethylmercury has neurological effects similar to methylmercury, but is considerably less toxic, with higher blood concentrations required to cause poisoning [112]. Although adverse effects have not been documented, thimerosal has recently been almost completely removed from U.S.-licensed vaccines to decrease systematic childhood exposure, but continues to be used in other countries [103].

93.4.1.2 Kinetics and Relation to Hair

The different chemical forms of mercury influence the absorption, distribution, toxicological manifestations, excretion, and useful methods of biological monitoring. Metallic elemental mercury, methylmercury, and ethylmercury all cross the blood–brain barrier. Elemental mercury also accumulates in the kidneys, and renal excretion is important in its elimination from the body [103,113].

Methylmercury penetrates into the central nervous system to a greater extent than ethylmercury due to the latter's larger size and faster decomposition, which may explain why ethylmercury is less neurotoxic than methylmercury [112].

Methylmercury is distributed widely and concentrated in the blood. Although methylmercury excretion is primarily fecal, it is also excreted into the hair where it accumulates and reaches concentrations ranging from 140 to 370 times that of blood [114]. The concentration in new hair is directly proportional to blood methylmercury concentration [115].

93.4.1.3 Indications for Hair Analysis

Properly handled hair samples are recognized biomarkers for methylmercury exposure in epidemiological studies using standardized protocols. This technique is well established and provides several advantages over blood mercury. In the clinical setting, problems may occur due to external contamination, use of hair samples far from the scalp, improper specimen handling, and the use of "commercial" hair tests [116,117]. Carefully collected samples analyzed by experienced research laboratories may provide additional corroboration of exposure or longitudinal information in some cases [118]. Hair analysis is not indicated to assess potential toxicity due to elemental mercury from dental amalgams, and hair results should not be used to justify unproven interventions such as amalgam filling, removal, and chelation.

93.4.2 Arsenic

93.4.2.1 Toxicology

The most common exposure to arsenic in the original population is from naturally occurring organoarsenates, primarily arsenobetaine and arsenocholine. These are concentrated in various types of sea food including fish and shellfish, but are innocuous. Health effects from arsenic are largely from tri- and pentavalent inorganic arsenic species with occupational exposures from byproducts of mining, smelting, and coal burning, and its use in various pesticides [119]. Worldwide, the most important exposures occur in discrete geographic areas with high levels of inorganic arsenic in the groundwater [120–123]. Chronic arsenic-related health effects are endemic in these populations.

Large acute exposures generally result from ingestions and produce an inflammatory gastroenteritis with altered vascular permeability that can produce hypovolemia and shock. Chronic arsenic exposure causes a wide variety of skin lesions including melanosis, keratosis and palmar and solar hyperkeratosis, as well as nonmelanoma skin cancers [120,123], also associated with peripheral vascular disease, neuropathy, hematopoetic disturbances, and lung cancer [120,122,123,119].

93.4.2.2 Kinetics and Relation to Hair

Arsenic is absorbed primarily through lungs and the gut [113]. It is distributed widely and rapidly cleared from the blood. Chronically, arsenic accumulates in the skin, hair, and nails due to their high content of sulfhydryl-rich keratin [113,124,125]. Other than some accumulation in the lungs, arsenic is not significantly stored in internal organs.

93.4.2.3 Indications for Hair Analysis

Hair arsenic can be a useful indicator of chronic arsenic poisoning in clinical and forensic settings, but its presence should only be considered as circumstantial evidence in most cases, unless external contamination can be ruled out [125]. In individual patients, hair levels must be correlated with clinical findings of arsenic toxicity, urine measurements, and possible exposure sources. Arsenic in hair has also been a valuable epidemiological tool in studies of individuals drinking arsenic-contaminated water when used along with urine, nail, and water arsenic measurements.

93.4.3 Lead

93.4.3.1 Toxicology

Lead is a xenobiotic. Exposures may occur during mining, smelting, and reflecting. It is the most widely utilized nonferrous metal and used in certain batteries, pigments, ammunition, and solders [126]. Average population exposures in countries that have eliminated leaded gasoline have dropped sharply in recent years [126,127]. The presence of lead paint in older homes remains, however, an important source of exposure to children and construction workers [126,128].

Lead is toxic to the blood, renal, reproductive, cardiovascular, and peripheral and central nervous systems [126,128]. Symptoms of lead toxicity in adults, if present, are varied and nonspecific, including abdominal pain, fatigue, headache, arthralgias, and myalgias. Central neurologic dysfunction depends on chronicity and severity and may range from subtle neurocognitive deficits to encephalopathy. Epidemiologically, increased lead body burdens otherwise not associated with disease in adults are associated with increases in blood pressure [129–131].

93.4.3.2 Kinetics and Relation to Hair

Absorption is highest through inhalation, but can also occur through the gastrointestitial tract, and is increased by iron and calcium deficiencies [113,126]. Once absorbed, lead is distributed widely throughout the body, where it exists in three compartments or pools [126]. The half-life in blood and other rapid exchange tissues is in the order of weeks, while in other soft tissues the half-life is measured in months [113]. Bone is the major endogenous storage site of lead, with

a half-life of 5–15 years [113,126]. Excretion is primarily renal, but small amounts are also excreted through the bile, hair, nails, and sweat [113].

93.4.3.3 Indications for Hair Analysis

Hair analysis for lead has limited epidemiological utility due to external contamination and inadequate sensitivity and specificity at lower levels of exposure, now identified as health concerns. It is not indicated for clinical diagnosis and not indicated for childhood or workplace screening programs. It may have limited applications in ecological and exposure assessment studies as a proxy marker of environmental contamination [132]. Anecdotally, segmental hair analysis was useful in corroborating the timeline of lead ingestion in a case of criminal poisoning [100].

93.4.4 Cadmium

93.4.4.1 Toxicology

Cadmium is a xenobiotic that occurs naturally with zinc and lead. Occupational uses include electroplating, cadmium alloy and battery production, welding solders, and cadmium pigments [133]. Smoking tobacco is the most important source of nonoccupational exposure to cadmium [113,133,134]. Large acute inhalational exposures can produce acute lung injury, while chronic exposures are nephrotoxic and epidemiologically linked to emphysema and bone demineralization [133].

93.4.4.2 Kinetics and Relation to Hair

Similar to lead, cadmium is absorbed well via inhalation and to a lesser extent through the gut [113,133]. Absorption is increased by calcium, iron, and zinc deficiencies. Cadmium is distributed throughout body tissues, with roughly 50% of the body load found in kidneys and about 15% in liver. Significant accumulation occurs in both anatomic sites with a biological half-life of 10–30 years in kidney and 5–10 years in liver [113,133]. Smokers have average twice the body load of nonsmokers [113,135]. Excretion is primarily renal, with lesser amounts excreted in the feces, saliva, hair, and nails [113].

93.4.4.3 Indications for Hair Analysis

At present, there are no forensic medicine indications for the measurement of cadmium in hair or as a biological marker of internal cadmium dose or body burden. It may have a role as a proxy marker of human interaction with environmental contamination.

93.4.5 Manganese

93.4.5.1 Toxicology

Manganese is an essential trace element that serves as a cofactor for several enzymes [136,137]. It also has a number of industrial uses including manganese alloys, dry cell batteries, paints, fertilizers, and in the gasoline additive, methylcyclopentadienyl manganese tricarbonyl (MMT) [136,137]. Occupational exposures also occur in mining and smelting. Manganese compounds are respiratory tract irritants [138]. Chronic inhalation exposure is associated with neuropsychiatric disturbances, with advanced neurological disease closely resembling Parkinsonism [136,137].

93.4.5.2 Kinetics and Relation to Hair

Occupational exposure occurs primarily from inhalation, but some gastrointestinal absorption also occurs. Manganese may share certain absorptive and metabolic pathways with iron [137,139]. The major storage site is the liver, but accumulation also occurs in the brain and kidney [113]. Excretion is primarily fecal, through the bile [137], but small amounts are also excreted in the urine, hair, sweat, and nail [113].

93.4.5.3 Indications for Hair Analysis

No single biological medium definitively and reliably reflects individual manganese exposure or toxicity. Therefore, hair manganese analyses should be considered as an additional source of potential exposure information in both individual cases and group studies, along with blood and urine manganese, and when possible environmental manganese determinations. The potential for external contamination must also be considered. Given the wide variation in reported background hair concentrations, control specimens should be obtained and analyzed using the same methods. Hair manganese is more likely to be informative regarding biological dose when the exposure is through ingestion rather than ambient air. In the latter case, the hair may be a surrogate measure of environmental manganese contamination.

93.4.6 Thallium

93.4.6.1 Toxicology

Thallium salts are toxic, and thallium sulfate has been used as a rodenticide. Although a 1970 ban on its use in the United States has reduced accidental poisonings, it continues to be used in some countries as a rodenticide, as well as being implicated in homicide attempts [140–142]. Anecdotal occupational exposure to thallium has also been described in the manufacture of a special glass [143].

Characteristically, the ingestion of thallium salts produces gastrointestinal symptoms, followed by the onset of a painful ascending neuropathy [140,141]. The neuropathy can be progressive with associated weakness. Acute alopecia begins after about a week, and can progress to complete baldness over several weeks [141]. Characteristically, the alopecia is painless, hair can be pulled out in clumps with little effort, and the inner one-third of eyebrows is spared [140,144,145]. In addition to diffuse alopecia, thallium intoxication can cause blackening of the hair roots, which is seen when these are examined microscopically [140,145].

93.4.6.2 Kinetics and Relation to Hair

Thallium is readily absorbed by inhalation, gastrointestinal absorption, and through the skin [141,142]. Subsequently,

thallium is widely distributed throughout the body and concentrated in the kidneys [141,142]. Excretion is predominantly renal and fecal, but thallium is also excreted into the saliva, nails, and hair [113,140]. The elimination half-life for thallium has been reported to be anywhere from 2 to 30 days [113,140,146]. Thallium does not have an internal anatomic reservoir, but binds sulfhydryl groups with high affinity like those in keratin, which likely explains its concentration in hair and nail [140,146].

93.4.6.3 Indications for Hair Analysis

Analysis of thallium content in hair should be considered as complementary to urine and blood testing in confirming the diagnosis of acute thallium poisoning, possible as early as 2 weeks after the onset of symptoms. Unlike urine samples, hair is unlikely to demonstrate elevated concentrations immediately after a single ingestion; however, given the relatively short elimination half-life of thallium, hair analysis may be especially useful in cases where several months have elapsed since the cessation of exposure. Analytic methods must be capable of quantifying concentrations in the parts per billion (ppb) range.

93.4.6.4 Commercial Hair Tests and Their Potential Misuses

Commercial hair analyses are promoted as a means of determining a patient's nutritional status and exposure to toxic heavy metals. Although hair analysis for heavy metals has a number of valid applications, as described earlier, commercial analyses that simultaneously determine large numbers of metals and minerals have long been misused.

The results are often used to justify questionable therapies such as extensive vitamin and other supplementation regimens, mercury amalgam filling, removal, and chelation [102,147–149].

Reliability study of commercial hair analyses using duplicate hair samples have demonstrated discordant reference range intervals, poor result reproducibility, and divergent nutritional recommendations from different laboratories analyzing identical samples [116].

Case reports of patients labeled as having heavy metal toxicity on the basis of commercial hair tests, which subsequently received second opinions, describe inaccurate diagnoses based on hair testing [97,102]. Such patients often experienced needless anxiety based on the inaccurate diagnoses. They also usually lacked significant exposure histories, and when conventional blood and urine tests were performed, they had normal concentrations of the metals in question.

A summary report of the U.S. Agency for Toxic Substances and Disease Registry (ATSDR) panel on hair analysis encapsulated the experts' view on commercial hair analyses with the following statement: "Universally, the panelists expressed concern about the misuse of hair analysis to justify and support unnecessary and unethical medical therapy" [99]. It is reasonable that in clinical situations, hair testing for heavy metals should be targeted to specific elements based on the patient's occupational and environmental history and exposure assessments, should be used only as an adjunct to more reliable biomarkers, and should utilize experienced reference or research laboratories.

93.5 HAIR IN FORENSIC TOXICOLOGY WITH A SPECIAL FOCUS ON DRUG-FACILITATED CRIMES

In the 1960s and 1970s, hair analysis was used to evaluate exposure to toxic heavy metals, such as arsenic, lead, or mercury. This was achieved using atomic absorption spectroscopy that allowed detection in the nanogram range. At that time, examination of the hair for organic substances, especially drugs, was not possible because analytical methods were not sensitive enough. Examination by means of drugs labeled with radioactive isotopes, however, established that these substances can move from blood to hair and be deposited there. Later, it was possible to demonstrate the presence of various organic drugs in hair by means of RIA. In 1979, Baumgartner et al. published the first report on the detection of morphine in the hair of heroin abusers using RIA. They found that differences in the concentration of morphine along the hair shaft correlated with the time of drug use. Today, gas chromatography coupled with mass spectrometry is the method of choice for hair analysis, and this technology is routinely used in forensic science to document repetitive drug exposure.

The major practical advantage of hair testing compared to urine or blood testing for drugs is that it has a larger surveillance window (week to month, depending on the length of the hair shaft, against from 2 to 4 days for most drugs in blood and urine). For practical purposes, the two tests complement each other. Urine analysis and blood analysis provide short-term information on an individual's drug use, whereas long-term histories are accessible through hair analysis.

Hair does not grow continually, but in cycles, alternating between periods of growth and quiescence. A follicle that is actively producing hair is said to be in the anagen phase. Hair is produced over 4–8 years at a rate of approximately 0.22–0.52 mm/month [150]. After this period, the hair follicle enters a relatively short transition period of about 2 weeks, known as the catagen phase, during which cell division stops and the follicle begins to degenerate. Following the transition phase, the hair follicle enters a resting or quiescent period, known as the telogen phase lasting approximately 10 weeks, in which the lower hair shaft forms a club root structure in the hair follicle with very low levels of metabolic activity. Factors such as race, disease states, nutritional deficiencies, and age are known to influence both the rate of growth and the length of the quiescent period. On the scalp of an adult, approximately 85% of the hair is in the growing phase, 2–3% in the catagen transition phase, and the remaining 12% in the telogen stage.

Pubic hair and axillary hair have been suggested as an alternative source for drug detection when scalp hair is not available. Various studies have found differences in drug

concentrations between pubic or axillary hair and scalp explained by a better blood circulation, a greater number of apocrine glands, a totally different telogen/anagen ratio, and a different growth rate of the hair, for instance, axillary hair 0.40 mm/day, and pubic hair 0.30 mm/day [150].

93.5.1 Mechanisms of Drug Incorporation into Hair

Drug can enter into hair by two processes: adsorption from the external environment and incorporation into the growing hair shaft from blood supplying the hair follicle. Drugs can enter the hair from exposure to chemicals in aerosols, smoke, or secretion from sweat and sebaceous glands. Sweat is known to contain drugs present in blood. Because hair is very porous and can increase its weight by up to 18% by absorbing liquids [151], drugs may be transferred easily into hair via sweat. Chemicals present in air, such as smoke, vapors, etc., can be deposited onto the hair.

Drugs appear to be incorporated into the hair shaft by at least three mechanisms: from the blood during hair formation, from sweat and sebum, and from the external environment. This model is more able than a passive model such as transfer from blood into the growing cells of the hair follicle to explain several experimental findings as described below:

- Drug and metabolites ratios in blood are quite different from those found in hair.
- Drug and metabolites concentrations in hair differ markedly in individuals receiving the same dose.

There is evidence for the transfer of the drugs via sweat and sebum at high concentrations and they persist in these secretions longer than they do in blood [151,152].

The extract mechanism by which chemicals are absorbed onto/absorbed into hair is not known. It has been suggested that passive diffusion may be augmented by drug binding to intracellular components of the hair cells such as the hair pigment melanin.

Another proposed mechanism is the binding of drugs with sulfhydrylcysteine in hair, which form cross-linking S–S bonds to stabilize the protein fiber network. Drugs diffusing into hair cells could be bound in this way.

93.5.2 Specimen Collection

Sampling and collection procedures for the analysis of drugs in hair have not been standardized. In most published studies, the samples are obtained from random locations on the scalp. Hair is best collected from the area at the back of the head, called the vertex posterior. Compared with the variability of the hair growth rate in other areas of the head, the number of hairs in the growing phase is more constant and the hair is less subject to age- and sex-related influences. Hair strands are cut as close as possible to the scalp, and their location on the scalp should be noted. Once hairs are collected, they may be stored at ambient temperature in aluminum foil, an envelope, or plastic tube. The sample size taken varies considerably among laboratories and depends on the drug to be analyzed and the test methodology. Sample size reported in the literature ranges from a single hair to 200 mg.

93.5.3 Stability of Drugs in Hair

The presence of opiates was detected in five hair shafts, which were about 7.5 cm in length, from the English poet John Keats 167 years after his death [153]. It is believed he took laudanum (opium) to control the pain of tuberculosis. The scalps of eight Chilean and Peruvian mummies dating from 2000 BC to AD 1500 also tested positive for benzoylecgonine, the main metabolite of cocaine [154].

The German composer Ludwig van Beethoven's hair was examined in four tests to study the cause of his many illnesses.

The first test was conducted by Dr. Werner Baumgartner at Osychemedics Corporation, Los Angeles, in May 1996. His radioimmuno assay experiment involved examination of 20 hairs to assess whether Beethoven received morphine, laudanum, or other opiates in final months of his life. Because Beethoven received care from Vienna's foremost physicians, it seems very likely that pain-relieving medication would have been offered to him, and that he might have declined it in order to be able to continue to sketch music, something he did until his final days.

The second test was performed in the fall of 1998 by Walter McCrone of Chicago's McCrone Research Institute. Using scanning electron microscope energy dispersion spectrometry, McCrone found lead levels in Beethoven's hairs that were 42 times higher than three control samples taken from living human beings. This result strongly indicated that Beethoven suffered from plumbism (lead poisoning) throughout much of his adult life even, dramatic lead toxicity that might explain his lifelong illness, his impacted personality, and even his death. However, it cannot be excluded that the hair had been contaminated during years of uncertain preservation.

In September 2000, Argonne National Laboratory physicists Ken Kemner, Derrick Manchini, and Francesco DeCarlo performed nondestructive synchrotron x-ray beam experiments involving side-by-side testing of six Beethoven hairs, a standard hair of known lead composition, and a standard "lead glass" film also of known composition.

The Argonne research team found elevated lead levels that averaged about 60 ppm in the six Beethoven hairs they examined, confirming McCrone's earlier finding.

Average Americans today have 0.6 ppm of lead in their hair, about 100 times less than the Beethoven hairs. Beethoven's lifelong illnesses such as terrible abdominal cramping, rheumatic fevers, abscesses, gouts, diarrhea, eye pain, and even his deafness may have been the result of severe lead toxicity.

Undetectable mercury levels were reported separately by the McCrone Research Institute and Argonne National Laboratory. These results exclude that Beethoven received

medical treatment for syphilis. In the 1820s, syphilis was normally treated with mercury compounds. This supports the consensus opinion of Beethoven scholars, who believe that Beethoven did not contract or suffer from syphilis.

To date, the owner of a flake of Beethoven's skull bone, which was removed from his remains when his body was exhumed in the 1860s, is not yet ready to publicize the results of similar tests performed on it, but the Argonne scientists who did the investigation ascertain that the bone testing has made them even more confident of the reliability of both their own and McCrone's findings in the hairs of Ludwig van Beethoven.

New evidence suggests the French emperor Napoleon Bonaparte did not die of cancer but was poisoned. According to two French forensic specialists in Strasbourg, tests on five strands of Napoleon's hair preserved since his death confirm "major exposure to arsenic."

Officially, he was said to have died of stomach cancer. According to Pascal Kintz, one of the two Strasbourg Forensic Institute's experts, "the level of arsenic found in Napoleon's hair is higher than 7–38 times normal amounts and is an unmistakable sign of poisoning." The analysis was commissioned by Ben Weider, a Canadian millionaire businessman and Napoleon enthusiast who has defended the poison theory for years. Mr. Weider, the founder of the International Napoleonic Society, recently received confirmative testing by an American laboratory of increasing arsenic concentrations in the emperor's hair 5 years ago. A year ago, he presented to French journalists his evidence and claims. Other theories were that it was a contamination from wallpapers in Napoleon's room on St. Helena, or that the local water was contaminated with arsenic. But the experts ruled this out, by stating that the high amounts found must have been deliberately administered.

All the studies indicate that drug information generated from analysis of hair is very stable in hair and by using chromatographic techniques, for example, archeologists may determine drug exposure as far back as to 2000 BC. Indians were already using cocaine. Organic substances are in some cases clearly capable of surviving in hair for thousands of years under favorable ambient conditions.

93.5.4 Application of Hair Analysis

By providing information on exposure to drugs over time, hair analysis may be useful in verifying self-reported histories of drug use and abuse in any situation in which a history of past rather than recent drug use is desired. In addition, hair analysis may be especially useful when a history of drug use is difficult or impossible to obtain.

Numerous forensic applications have been described in the literature where hair analysis was used to document the case: differentiation between a drug dealer and a drug customer, chronic poisoning, crime under the influence of a drug, child sedation and abuse, suspicious death, child custody, abuse of drugs in jail, body license, and doping control [155–157].

More than 500 articles concerning hair analysis have been published to date reporting applications in forensic toxicology, clinical toxicology, occupational medicine, and doping control. The major practical advantage of hair for testing drugs, compared with urine or blood, is its larger detection window timewise, which is counted in weeks to months, depending on the length of hair shaft analyzed, against a few days for urine. In practice, detection windows offered by urine and hair testing are complementary: urine analysis provides short-term information of an individual's drug use, whereas long-term histories are accessible through hair analysis. Although there is reasonable agreement that the qualitative results from hair analysis are valid, the interpretation of the results is still under debate owing to unresolved questions such as the influences of external contamination or cosmetic treatment, and possible genetic differences.

Further researches should be required before all of the scientific questions associated with hair drug testing will be satisfied. There is a lack of consensus among the active investigators on how to interpret the analysis of drugs in hair. Among the unanswered questions, five are of special importance:

- What is the minimal amount of drug detectable in hair after administration?
- What is the relationship between the amount of the drug used and the concentration of the drug or its metabolites in hair?
- What is the influence of hair color?
- What is the influence of genetic differences in the hair being tested?
- What is the influence of cosmetic treatment?

Several answers were recently addressed by some reports [158,159] on these specific topics.

93.5.5 Special Focus on Drug-Facilitated Crimes

The use of a drug to modify a person's behavior for criminal gain is not a recent phenomenon. However, the sudden increase in reports of drug-facilitated crimes (sexual assaults, robbery, etc.) has caused concerns in the general public. Drugs involved can be pharmaceuticals, such as benzodiazepines (flunitrazepam, lorazepam, etc.), hypnotics (zopiclone, zolpidem), sedatives (neuroleptics, some histamine H1 antagonists), anesthetics (gamma-hydroxybutyrate or GHB, ketamine), drugs of abuse (cannabis, ecstasy, D-lysergic acid dimethylamida (LSD)), or more often ethanol. Most of these substances possess amnesic properties and in sex offences, for example, the victims are less able to accurately recall the circumstances under which the offence occurred, as they impair an individual rapidly. Because of their low dosage, except for GHB, a surreptitious administration into beverages such as coffee, soft drinks (cola), or even better alcoholic cocktails is relatively simple.

To perform successful toxicological examinations, the following important rules should be followed:

- To obtain as soon as possible the corresponding biological specimens (blood, urine, and hair)
- To use sophisticated analytical techniques (LC/MS, headspace/GC/MS, tandem mass spectrometry)
- To take care in the interpretation of the findings (LC/MS; liquid chromatography/mass spectrometry)

As is the case with applications such as survey of addicts, doping control, driving license regrant, etc., hair testing is a valuable approach to increase the window of drug detection. Embarrassment associated with urine collection, particularly after sexual assault, can be greatly mitigated through hair analysis. It is always possible to obtain a fresh identical hair sample if there is any trouble during analysis, for example, a specimen mix-up or a breach in the chain of custody. This makes hair analysis essentially fail-safe, in contrast to blood or urine analysis, since an identical blood or urine specimen cannot be obtained at a later date.

The discrimination between a single exposure and long-term use can be documented by multisectional analysis. With the concept of absence of migration along the hair shaft, a single spot of exposure must be present in the segment corresponding to the period of the alleged event, using a growth rate for hair of 1 cm/month. As this growth rate can vary from 0.7 to 1.4 cm/month, the length of the hair section must be calculated accordingly. A delay of 3–4 weeks between the offence and hair collection and analysis of 2-cm sections is considered satisfactory to include the spot of exposure in the hair shaft sample. The hair must be cut as close as possible to the scalp. Particular care is also required to ensure that the individual's hair strands retain the positions they originally had beside one another.

The unique possibility to demonstrate a single drug exposure through hair analysis has some additional interests. In case of late crime declaration, positive hair findings are of paramount importance for a victim, in order to start, under suitable conditions, a psychological follow-up. It can also help in the discrimination of false reports of assault, for example, in the case of revenge. These cases are often sensitive with little other forensic evidence. Tedious interpretations, for example, in case of concomitant intake of hypnotics as a therapy for sleeping disorders, are avoided when investigations are done using hair instead of urine. Finally, in the absence of hair testing, it is always possible for the advocate of the defendant to claim during the criminal trial that the detected drug was ingested by the victim himself/herself and had no connection with the crime.

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94 Popliteal Lymph Node Assay

Guillaume Ravel and Jacques Descotes

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94.1 INTRODUCTION

The popliteal lymph node assay (PLNA) was initially proposed to assess new molecular entities for the potential to induce autoimmunity [1] or, to a much lesser extent, immunosuppression [2]. Although autoimmunity is still a central issue, the PLNA is also being increasingly considered as a tool for predicting drug allergies [3].

The PLNA was introduced by the seminal paper of Helga Gleichmann, who showed that the injection of the anticonvulsant drug phenytoin into a hind footpad of mice significantly increased the weight of the draining PLN compared with the contralateral untreated PLN [4]. The findings in animal models of graft-versus-host (GvH) reactions and the clinical signs and symptoms of phenytoin hypersensitivity in man are strongly reminiscent of a GvH disease [5]. Gleichmann et al. [6] hypothesized that GvH-like T-cell reactions could be mounted toward adjacent lymphocytes rendered histoincompatible following phenytoin administration. As Ford et al. [7] had previously shown that the injection of histoincompatible lymphocytes into the footpad of rats produced a regional GvH reaction evidenced by PLN enlargement, the induction of increased PLN weight by phenytoin injection to mice lent support to the (pseudo-)GvH hypothesis of phenytoin hypersensitivity and paved the way for further development of the PLNA [8,9].

Over the years, a number of investigators have proposed refinements or modifications of the initial procedure and four main experimental designs of the PLNA predominate at the present time.

94.2 THE DIRECT (OR PRIMARY) PLNA

The direct PLNA is the most extensively used and is the procedure that has been partly validated.

94.2.1 Standard Procedure

The direct PLNA is rapid, simple, and inexpensive [10]. The test article is injected subcutaneously into one hind footpad between the heel and the toes. The same volume of the vehicle is injected into the opposite hind footpad. Both PLNs are removed, usually on day 7. They are carefully dissected from the surrounding tissue and immediately weighed (Figure 94.1).

Although mice or rats can be used, dissection of the PLN in mice requires more technical skill. Balb/c and C57B1/10 mice have once been suggested to give greater responses than DBA/2 mice [8], whereas other workers found the following order of decreasing responses to the antidepressant zimeldine: C56Bl/6 > DBA/2 > Balb/c mice [11]. Finally, in another study comparing Balb/c, A/J, and ICR mice, only small interstrain differences were noted [12]. In recent years, Balb/c mice have been the most used. Brown-Norway rats have been reported to produce greater PLN responses than Sprague-Dawley rats [9], but this was not confirmed in a further study comparing the responses to streptozotocin and phenytoin in the Wistar, Wistar-Furth, Sprague-Dawley, Fisher 344, Lewis, and Brown-Norway strains [13].

The injection volume is 10–50 µl per mouse or rat. No results are available to show whether this volume has any influence on the magnitude of the responses. Saline is the preferred vehicle, but other vehicles have to be considered for poorly hydrosoluble drugs or chemicals. Suitable solvents include 20% DMSO and 25% ethanol, but acetone, methyl ethyl acetone, and pure ethanol produce false-positive responses due to primary irritation [14,15]. Corn oil, paraffin oil, and 1,2-propylene glycol are also unsuitable solvents, as they produce false-positive responses probably due to their excessive viscosity [15].

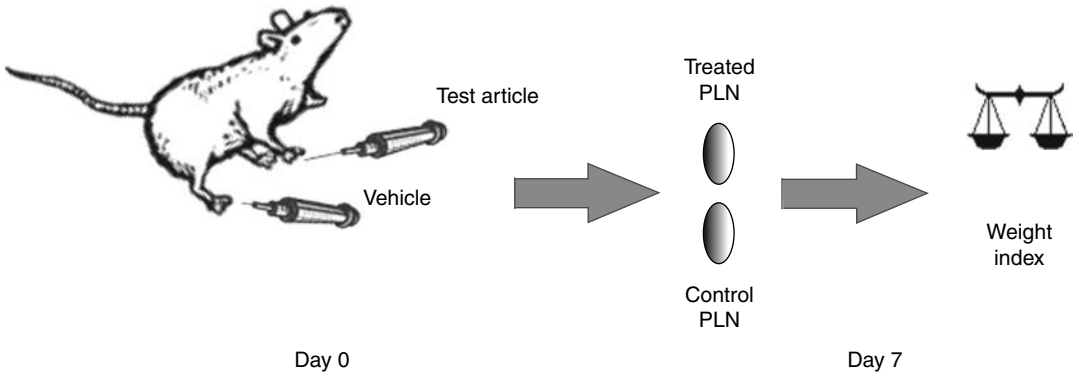


FIGURE 94.1 The direct (primary) PLNA. The test article is injected subcutaneously into one hind footpad of a mouse or rat, and the same volume of the vehicle into the opposite footpad. Both PLNs are removed on day 7, and then carefully dissected from surrounding tissue and immediately weighted to calculate the weight index.

The administered dose is arbitrarily 1 mg per mouse and 5 mg per rat in most instances. Interestingly, a dose of up to 5 mg of procainamide per mouse was shown to give a positive response in contrast to a dose of 1 mg [16]. A dose of 0.3 mg zimeldine per mouse induced a negative PLN response, whereas a dose of 1 mg induced a positive response [11]. However, increasing the dose can also result in systemic toxicity as shown with mercuric chloride [15]. No relationship between the dose and the resulting PLN weight increase was found with phenytoin, streptozotocin, sulfamethoxazole, ofloxacin, phenobarbital, and metformin in rats [17]. In contrast, increasing the dose of mercuric chloride, hydralazine, and streptozotocin resulted in a greater increase in murine PLN cellularity [18]. Similarly, the injection of 5 mg penicillin G induced a negative response in Brown-Norway rats [19] in contrast to several strains of mice [12]. Whether differences in species or dose account for these results is not known.

Both PLNs are immediately weighed following dissection to calculate the weight index (WI), that is, the ratio of the treated versus control PLN weights. There is no general agreement on the definition of a positive response. Two methods have been used. One method is based on the calculation of the upper limit of the 5% confidence interval using the historical control database of the testing laboratory. Another method consists of the statistical pairwise comparison of the weight of the treated versus control PLN in each animal. In fact, neither of these approaches is considered optimal. In practice, a nominal value of 2 is often applied to define a positive WI. In our experience, the upper limit of the confidence interval is always <2. WI values of only slightly >1 may attain statistical significance in pairwise tests [19]. Therefore, the nominal threshold of 2 is a more robust limit of positivity. A WI of more than 10 is seldom observed. A systemic reaction may, rarely, result in increased weight of the contralateral (control) PLN (unpublished results), so the use of an additional group of animals injected with the vehicle in both hind footpads may be necessary when the control PLN weight in the standard assay falls outside the expected range.

Although WI is often considered as the gold standard in the PLNA, the magnitude of the response can also be assessed from the cellularity index (CI). Immediately after weighing, the PLNs are gently crushed and centrifuged to obtain a suspension of the recovered PLN cells, which are counted either microscopically or using a FACS scan. CI is defined as the ratio of the treated versus control PLN cell count in each animal. CI is most often greater than WI. Again there is no widely accepted definition of a positive CI, and the same methods as those described for WI can be used. A positive response is often defined as $CI > 5$. CI values up to 50 have been observed. CI has been suggested as a more sensitive endpoint in the direct PLNA, since $CI > 5$ may be seen in animals with $WI < 2$ [19].

As indicated above, PLNs are typically removed 7 days after injection. However, delayed stronger responses can occur, as reported with zimeldine [11] and streptozotocin [20], even though positive responses were already present on day 7 in both instances. Kinetic studies of PLN responses may be helpful in ruling out false-negative responses, but this has yet to be assessed.

94.2.2 Refinement of the Standard Procedure

As already suggested, false-positive and false-negative responses demonstrate suboptimal sensitivity and specificity in the direct PLNA. Various refinements to the standard procedure have been proposed.

(False) negative responses in the direct PLNA have been obtained with medicinal products, such as procainamide and isoniazid, that are known to induce systemic autoimmune reactions in human subjects. These false-negative responses often appear to be due to the involvement of reactive metabolites instead of the parent molecule. Although metabolic activity including cytochrome P450 is present in PLNs, an insufficient amount of the reactive metabolites may be produced in the standard procedure to obtain positive responses. As already mentioned, increasing the dose has proven successful in obtaining positive responses with procainamide [16]. Another method is activation with

S9-mix fractions *in vitro* prior to injection into the footpad, as shown with procainamide [21] or isoniazid [22]. Similarly, pretreatment of rats with the enzyme inducers β-naphthaflavone or phenobarbitone led to positive responses with procainamide [22].

Whereas false-negative responses are seemingly rare and rather easily ruled out as described above, false-positive responses may be relatively frequent. These may be caused by specific immune responses. Standard histological examination was included early as an endpoint in the direct PLNA [23,24]. Frank positive responses are characterized by a more or less pronounced blurring of the normal lymph node architecture with intermingling of the cortical and paracortical areas, numerous immunoblasts in the paracortical area, high-grade maturation of the germinal centers, and numerous plasma cells in the medullary area. These changes closely mimic a GvH reaction and were considered to support the pseudo-GvH hypothesis as the mechanism involved in PLN responses. Positive responses have been obtained with potent contact sensitizers, such as dinitrochlorobenzene (DNCB) and picryl chloride [23,24], or following injection of direct immunogens, such as sheep erythrocytes into the footpad. In both cases, standard histological examination reveals changes markedly dissimilar to those seen in a true positive response, in particular a conserved architecture of the lymph node and numerous germinal centers [24].

Another major cause of false-positive responses in the PLNA is primary irritation. Standard histology proved unable to detect false-positive responses due to primary irritants [24]. Immunohistochemistry may be useful for this purpose, but has been rarely used to date. The amount of tritiated thymidine incorporated by PLN cells was shown to correlate with WI following the injection of phenytoin, streptozotocin, sulfamethoxazole, ofloxacin, phenobarbital, or metformin to mice [17]. However, neither the sensitivity nor the specificity of the assay was improved. Lymphocyte subset analysis by flow cytometry has seldom been included in the direct PLNA. An early study evidenced a global increase in the numbers of B, T, $CD4^+$, and $CD8^+$ T cells that paralleled the increase in CI [25]. The subset analysis of PLN cells from mice 4, 5, 6, and 12 days following injection of streptozotocin [18] demonstrated an initial fall in $CD4^+$ T cells followed by a subsequent increase on day 6, which persisted to day 12. An opposite trend was seen for $CD8^+$ T cells. There was also an early, but transient increase in both $CD25^+$ and $CD69^+$ T cells. In contrast, mercuric chloride induced only slight changes and hydralazine caused a small reduction in $CD4^+$ T cells. More specific T-cell markers may be expected to generate useful information, but so far none have been applied to the direct PLNA.

Whatever the method used, the elimination of false-positive responses induced by primary irritants was largely unsuccessful. The depletion of T cells by pretreatment of mice with monoclonal antibodies against $CD4^+$ or $CD8^+$ T cells is, however, an interesting approach [26]. False-positive responses to the primary irritants imipramine, acetone, and ethanol were observed in normal mice as well as in mice depleted in either $CD4^+$ or $CD8^+$ T cells. (True) positive responses to streptozotocin were seen in mice depleted in $CD4^+$ T cells, but not in mice depleted in $CD8^+$ T cells [27,28]. This suggests that $CD8^+$ T cells play a critical role in genuinely positive PLN responses.

Finally, the production of cytokines has been suggested as a potentially useful endpoint in the direct PLNA. In mice, streptozotocin induced a dramatic increase in interferon-γ (IFN-γ) mRNA production that correlated with increases in WI and CI, but no change in the production of interleukin 4 (IL-4) mRNA [29]. Subsequently, streptozotocin was shown to induce a marked increase in the production of IL-6, IFN-γ, tumor necrosis factor alpha (TNF-α), IL-1α, IL-1β, IL-2R, and IL-12 mRNA [26]; whereas neither ethanol nor acetone induced changes in the production of IL-6 and IFN-γ mRNA [28]. In addition, increases in the production of TNF-α, IL-1α, IL-1β, IL-2R, and IL-12 mRNA were two to three times lower in mice treated with acetone or ethanol than with streptozotocin. Tuschl et al. [18] found increased intracellular PLN levels of IL-2 and IFN-γ following streptozotocin injection. However, the reliability of cytokine production measurement in the direct PLNA was subsequently questioned, since neither imipramine, glafenine—an analgesic withdrawn from the market because of anaphylactic shock—nor hydralazine and minocycline, which have repeatedly been reported to cause systemic autoimmune reactions, induced any changes in the production of the proinflammatory cytokines IL-6, TNF-α, the T_{H1} cytokines IL-2, IL-12, and IFN-γ, or the T_{H2} cytokines IL-4 and IL-5 [30].

94.3 THE SECONDARY PLNA

Since the direct PLNA does not provide evidence of whether a positive response is antigen-specific, the secondary PLNA was proposed to test for T-lymphocyte sensitization (Figure 94.2). The major difference with respect to the direct (primary) PLNA is that the secondary PLNA is a two-step procedure [31]. The first step is identical to the direct PLNA with a subcutaneous injection of the test article into one hind footpad, and the same volume of the vehicle into the contralateral footpad. The same rules and uncertainties apply with regard to the injected dose of the test article, the volume of administration, or the nature of the solvent. To the best of our knowledge, the secondary PLNA has only been used in mice. The second step is performed after a rest period of 4–6 weeks to allow for total resolution of any primary response. The same (primed) animals are injected subcutaneously into the same footpad with the test compound. The animals are sacrificed 4–6 days after challenge and the weight and cellularity indices are calculated as described above. The challenge dose of test compound administered in the second step is set lower than the lowest dose capable of inducing a positive response in the first step, thus allowing the

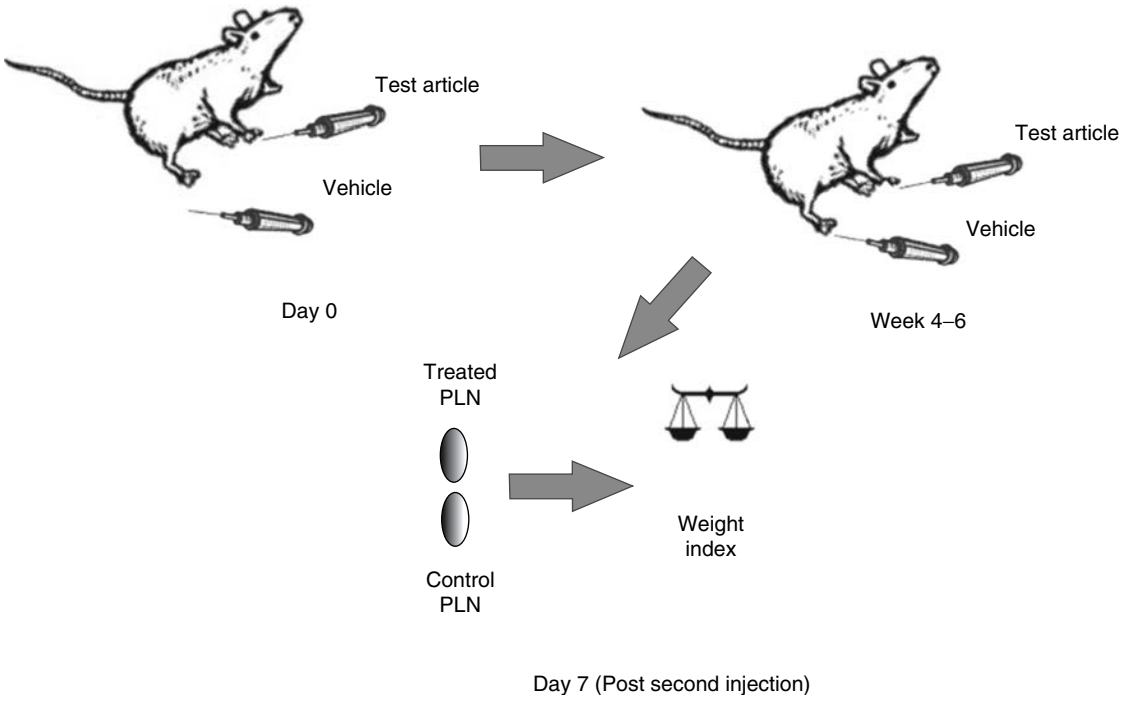


FIGURE 94.2 The secondary PLNA. The test article and the vehicle are injected into the footpads as in the direct PLNA. After a rest period of 4–6 weeks, the same animals are injected with a lower dose of the test article into one footpad and the same volume of the vehicle into the opposite footpad. Seven days later, both PLNs are removed, dissected from surrounding tissue, and immediately weighted to calculate the weight index.

differentiation of T cell-mediated memory responses from irritant responses [32].

94.4 THE ADOPTIVE PLNA

Like the secondary PLNA, the adoptive PLNA is a two-step procedure (Figure 94.3). Mice are given one or several administrations of the test article (or one of its metabolites) via the subcutaneous route, for example, on the dorsum, or via the intranasal [33], oral [34], or intravenous [35] routes. Irradiated splenic T cells from these mice are then injected into the hind footpad of naïve syngeneic mice 1 day before injection of the test article (or metabolite) into the same footpad. The animals are sacrificed 4–6 days after challenge, and the weight and cellularity indices are calculated.

The major advantage of the adoptive PLNA is its ability to demonstrate the involvement of a specific T-cell response triggered by the test article or one of its metabolites. The antithyroid drug propylthiouracil, which has been repeatedly reported to cause systemic autoimmune reactions in human subjects, induced a negative response in the direct PLNA in contrast to its reactive metabolite propyluracil 2-sulfonate. Interestingly, a strong secondary response was observed in the adoptive PLNA with propyluracil 2-sulfonate, but not with propylthiouracil, following injection of spleen cells from mice pretreated with the corresponding compound [35]. Oxidation of Au^{+} to the reactive intermediate Au^{3+} is involved in the immune-mediated adverse effects of the antirheumatic drug gold (Au^{+}) disodium thiomalate. The injection of spleen cells from mice pretreated with either a single injection of tetrachloroauric (Au^{3+}) acid or with 12 weekly intramuscular injections of gold disodium thiomalate led to an amnestic response following subsequent challenge with Au^{3+} injection into the footpad [36]. Similarly, neither procainamide nor *N*-acetyl-procainamide induced a positive response in the direct PLNA, in contrast to the reactive metabolite hydroxylamine-procainamide. The reaction was shown to be specific to hydroxylamine-procainamide by injecting spleen cells from mice given three injections of hydroxylamine-procainamide: a secondary response was observed with hydroxylamine-procainamide, but not with procainamide or *N*-acetyl-procainamide [37].

On the basis of these results, the adoptive PLNA may be considered as a useful tool to evidence the role of specific T-cell responses, and also to rule out a false-negative response due to the involvement of metabolites instead of the parent molecule. However, the use of large numbers of animals and the sophisticated technical skills required are major limitations to the implementation of the adoptive PLNA as a routine assay.

94.5 THE MODIFIED PLNA

In a further attempt to investigate immune mechanisms involving T-cell activation in PLN responses, Pieters et al. [38,39] developed the modified PLNA (Figure 94.4).

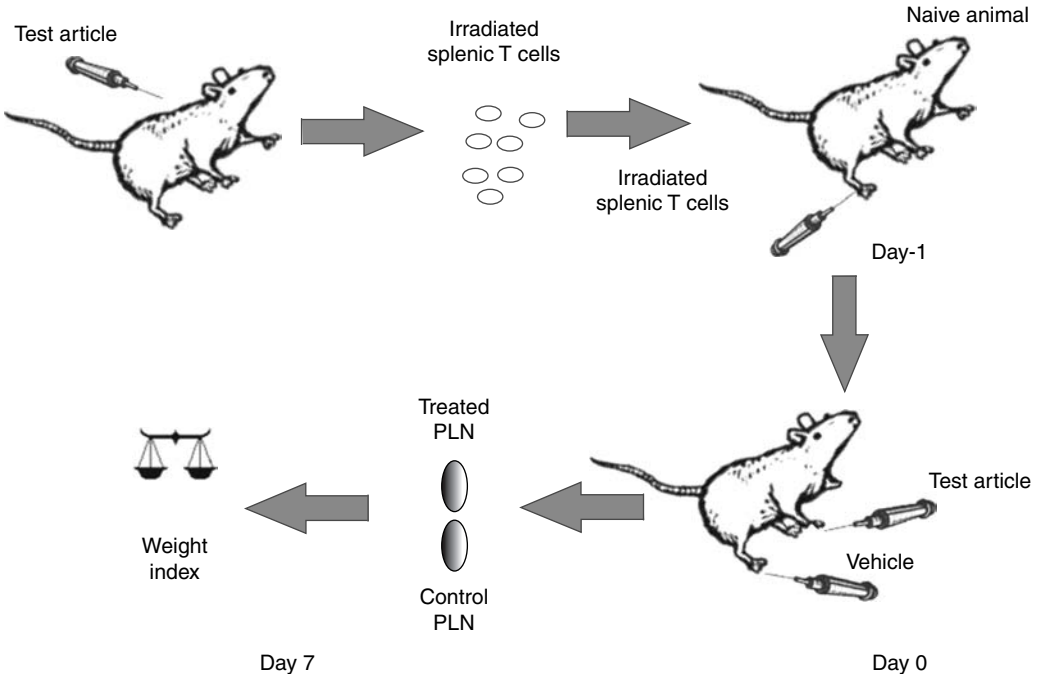


FIGURE 94.3 The adoptive PLNA. Mice are given one or several administrations of the test article by the subcutaneous, intranasal, oral, or intravenous route. Spleen T cells from these mice are irradiated and then injected into the footpad of naïve syngeneic mice on the day before the injection of the test article and vehicle into the footpad as in the direct PLNA. Seven days later, both PLNs are removed, dissected from surrounding tissue, and immediately weighted to calculate the weight index.

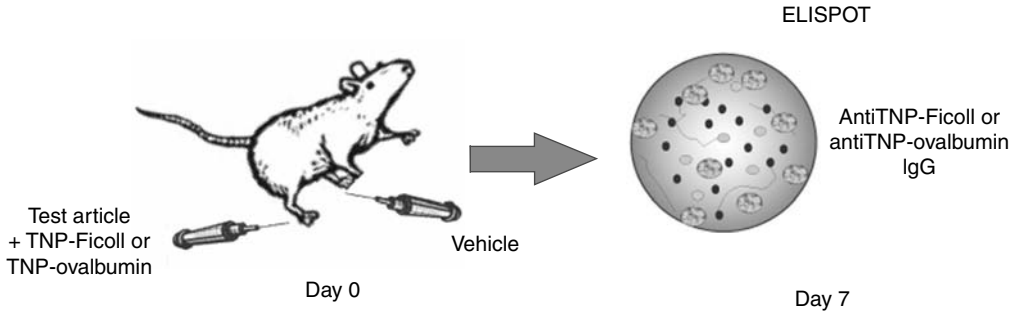


FIGURE 94.4 The modified PLNA. Mice are injected into the footpad with the test article and the reporter antigen TNP–Ficoll or TNP–ovalbumin. The specific antibody response is measured by ELISPOT 7 days later.

To characterize the immunostimulatory or sensitizing potency of the test compound, a local specific antibody response to the reporter antigens TNP–Ficoll (2,4,6-trinitrophenyl) and TNP–ovalbumin is elicited. The test article, together with 10 μg per mouse of either TNP–Ficoll or TNP–ovalbumin, is injected into one hind footpad of mice. Specific antibody responses are measured 7 days later by ELISPOT. An inflammatory reaction is characterized by a positive IgG response to the T-dependent antigen TNP–ovalbumin, but not to the T-independent antigen TNP–Ficoll. A T cell-mediated reaction is characterized by a positive IgG response to both TNP–ovalbumin and TNP–Ficoll. Positive responses result in a 10- to 1000-fold increase in cells producing anti-TNP–Ficoll IgG versus a 20-fold increase in cells producing anti-TNP–ovalbumin IgG. Therefore, the potential for discriminating sensitizing from nonsensitizing compounds is theoretically high, and even allows the ranking of compounds according to their immunostimulatory potency. In addition, the IgG1 and IgG2a may be assayed to assess T_{H2} responses and IgE to assess T_{H1} responses. Indeed, streptozocin was shown to induce an IgG2a or IgG2b response associated with IFN-γ release; whereas diclofenac, zomepirac, and glafenin (which have been reported to cause anaphylactic, but not systemic autoimmune reactions in humans) induced an IgG1 or IgE response and IL-4 release [40]. However, mercuric chloride [41] as well as high doses of zomepirac and glafenin [42] produced a mixed IgG1, IgE, IgG2a, and IgG2b response in this system.

94.6 FUTURE DIRECTIONS

From the above it is clear that the PLNA is not yet sufficiently well defined to be recommended for routine use in the preclinical safety evaluation of drugs and chemicals.

The primary (direct) PLNA is the only procedure that has been at least partly validated. Consistent results have been obtained for the blind assessment of chlorpromazine, zimeldine, hydrazine, streptozotocin, and barbital in three different laboratories using strictly the same procedure in the rat [43]. Although it is correct that over 100 drugs and chemicals have been tested in the PLNA [44], it is important to keep in mind that the vast of majority of results have been obtained only in the primary (direct) PLNA. In fact, very few compounds, for example, the antitumor drug streptozotocin and mercuric chloride, have been tested repeatedly in a number of different laboratories. Streptozotocin has been shown to induce immunological and histological changes in the PLN of treated mice, which closely resembles an acute GvH reaction [20,45]; there is an early and marked production of IFN-γ by $CD8^+$ T cells involving the D-glucopyranose moiety of streptozotocin [26,46]. This direct immunostimulatory effect is thought to account for the positive PLN responses, but neither an autoimmune nor an allergic mechanism is actually involved. Conflicting results with mercuric chloride have been obtained in the PLNA [15,18,38,42], and it is noteworthy that mercuric chloride has never been conclusively demonstrated to induce autoimmune diseases in man. Therefore, the selection of adequate reference compounds for further validation of the PLNA is absolutely essential.

Our current understanding of the mechanism(s) involved in the PLNA is poor. The initial concept of pseudo-GvH mechanism to explain PLN responses produced by drugs and chemicals that have been reported to cause systemic autoimmune-like reactions in human subjects is supported by histological and immunological findings [4,20,23,24], but no definitive evidence has so far been provided. Activated T cells certainly play a pivotal role in PLN responses, but activation can result from a direct immunopharmacologic effect (as with streptozotocin [46–49]), a nonspecific mechanism (e.g., macrophage activation [50]), a nonspecific inflammatory response [28], or an antigen-specific mechanism [31,33–37]. Since quite different mechanisms result in PLN weight increase, the PLNA lacks the required sensitivity and specificity for use in current routine preclinical safety evaluation. Our poor understanding of mechanisms largely explains the relatively diverging objectives of current PLNA research. The primary (direct) PLNA was initially developed as a tool to predict drug-induced systemic autoimmune reactions [1,51]. Nowadays, the focus is on the prediction of sensitization or drug allergies, which is somewhat confusing [3].

Despite all current uncertainties and discrepancies, the available data suggest that the modified PLNA may be most suitable for the identification of sensitizing drugs with the potential to induce anaphylaxis in humans, as well as immunostimulatory drugs. Inconsistent results have been obtained with diclofenac, zomepirac, and glafenine in the direct PLNA, whereas a marked antiTNP–ovalbumin IgE response was seen in the modified PLNA [42]. In contrast, the modified PLNA does not seem to be suitable to detect medicinal products with the potential for inducing systemic autoimmune reactions in humans, since neither hydralazine nor isoniazid and α-methyldopa gave positive responses in this assay [42]. The primary (direct) PLNA remains a potentially useful tool to predict the potential for drug-induced systemic autoimmune reactions. Importantly, most drugs that induced positive responses in this assay have been reported to cause the drug hypersensitivity syndrome DRESS (drug rash with eosinophilia and systemic symptoms). DRESS is a severe T-cell-mediated cutaneous drug reaction with fever, lymphadenopathy, and varied visceral involvements (hepatitis, pneumonitis, myocarditis, pericarditis, nephritis, etc.) [52]. The signs and symptoms described in many cases of drug-induced systemic autoimmune reaction mimic those of DRESS, and not those of spontaneous systemic autoimmune diseases. The primary (direct) PLNA may be considered best suited for the detection of such adverse reactions that possibly involve direct interactions between medicinal products and T cells [53], as suggested with streptozotocin [46].

In conclusion, advances in our understanding of underlying mechanisms, further evaluation using well-defined endpoints, and extensive validation are needed to assess the relevance of the PLNA for the identification of drugs and chemicals with the potential to induce hypersensitivity and systemic autoimmune reactions in humans.

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95 Pigmentation Changes Resulting from Arsenic Exposure

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95.1 INTRODUCTION

Arsenic, the 20th most abundant element in the earth's crust, is present in trace levels in all soils, rocks, waters, and air. Arsenic compounds can exist in both inorganic and organic forms and are classified according to their valence states: elemental (0), arsenite (trivalent, 3+), and arsenate (pentavalent, 5+). Inorganic arsenic is released into the atmosphere and natural waters from weathering and dissolution of arsenic-containing minerals, volcanic activity, microbial activity, and anthropogenic activities.

Arsenic can be present at high concentrations in nonferrous ores including copper, zinc, and gold and is a common contaminant of coal. Biological activity can transform inorganic arsenic into organic forms. Seafood can contain high concentrations of organic arsenic but these species are considered to be nontoxic.

The biological effect of arsenic will largely depend on the arsenic species, dose, and duration of exposure. The International Agency for Research on Cancer has determined that there are sufficient human data to classify inorganic arsenic as a known human carcinogen (IARC 2004).

95.2 SOURCES OF EXPOSURE TO ARSENIC

Humans can be exposed to arsenic from environmental, medicinal, and occupational sources, as well as from intentional or accidental poisoning.

95.2.1 Environmental Exposure

Consumption of arsenic-contaminated drinking water is the primary route of exposure for most individuals. In many countries, aquifers pass through arsenic-rich geological strata resulting in groundwater with elevated arsenic levels. Most notably affected by arsenic-contaminated groundwater are Bangladesh, West Bengal, India, and Inner Mongolia, China.

Surface water can also become contaminated from anthropogenic sources. For instance, arsenic concentrations in acid mine drainage can reach very high concentrations. Arsenic concentrations are also high in the geothermal water in Kamchatka, Japan, Alaska, and California (Nordstrom 2002).

Bottled water can also contain arsenic if the source water comes from a contaminated aquifer. For instance, Armenian

mineral water "Jermuk" was found to contain arsenic at concentrations ranging up to 600 μg/L. This popular mineral water has been widely available in Armenia and Russia for decades.

Food grown in contaminated soil or irrigated with contaminated water can accumulate arsenic. Total dietary studies have shown that rice and cereal grains are the primary source of inorganic arsenic in the diet (Yost et al. 1998; Tao and Bolger 1999; Schoof et al. 1999). Seafood contains high concentrations of organic arsenic including arsenocholine and arsenobetaine, commonly called "fish arsenic," which are considered to be nontoxic (Kaise et al. 1985). Thus, an individual's dietary exposure will depend on his/her food habits and the agricultural origin of the food.

95.2.2 Medicinal Exposure

Arsenic has a long history in medical and veterinarian medicines. In 1786, Thomas Fowler developed a solution of 1% potassium arsenite, which now bears his name. Fowler's solution was touted as a general tonic for ailments ranging from jealousy to cancer and was most likely the first effective treatment for leukemia (Waxman and Anderson 2001). Arsenic's antimicrobial properties were exploited by Paul Ehrlich in 1907, who synthesized Salvarsan (3,3′-diamino-4,4′-dihydroxyarsenobenzene dihydrochloride) (Schwartz 2004). Both substances were phased out of use by the mid-1900s due to the development of more effective treatments and concerns about carcinogenic risks.

Currently, arsenic trioxide (As_2O_3) is used to treat acute promyelocytic leukemia (Shen et al. 1997) and as a treatment for trypanosomiasis (Finch and Snyder 1982). It can also be an ingredient in folk remedies, particularly of Asian origin (Ernst 2000). A survey conducted in Boston of commonly available South Asian Ayurvedic homeopathic medicines found arsenic concentrations ranging from 37 to 8130 μg/g, which if taken regularly could result in arsenic toxicity (Saper et al. 2004).

95.2.3 Occupational Exposure

In 2004, the annual world production of arsenic is estimated to be 37,000 tons with 75% of the production occurring in China, Chile, and Peru. In addition to the extraction and refining of arsenic ores, occupational exposures most often occur in smelting, mining, glass manufacturing, and semiconductor industries.

Some coal deposits can also have elevated arsenic concentrations depending on localized geological factors. Coal miners in the Guizhou Province of China are at particular risk because coal deposits in this region contain very high concentrations of arsenic. In this region, it is common for coal to be burned inside the home for cooking and crop drying purposes resulting in chronic arsenic exposure from contaminated indoor air and food (Liu et al. 2002).

Woodworkers can be exposed to inorganic arsenic from wood treated with chromium, copper, and arsenic (CCA), a common preservative. These workers can have dermal exposure from improperly cured wood or inhalation exposure from breathing sawdust (Peters et al. 1986; Decker et al. 2002).

Inorganic arsenic is also added as an active ingredient in some commercially prepared insecticides, herbicides, and rat poisons. Inorganic arsenic-based herbicides including lead arsenate, calcium arsenate, and sodium arsenate, once widely used in agriculture, have largely been banned in Western countries out of concern for worker safety. However, herbicides containing organic arsenicals such as monosodium methanearsonate (MSMA), disodium monomethylarsonate (DSMA), calcium acid methanearsonate (CAMA), and cacodylic acid have taken their place and are currently used in cotton agriculture and in lawn care.

95.2.4 Intentional or Accidental Poisoning

In the middle ages, arsenic was a well-known poisoning agent because it was tasteless, odorless, fatal at very low doses, and incurable. Its popularity as an agent of assassination declined only in the nineteenth century when forensic medicine developed reliable detection methods. In the modern era, accidental poisoning or attempted suicide is the most common reason for acute arsenic poisoning.

A notable example of accidental poisoning occurred in Manchester, England in the early 1900s, which afflicted approximately 6000 beer drinkers and resulted in 70 fatalities (Brooke and Roberts 1901). The source of the arsenic contamination was traced to the glucose, or brewer's sugar, made from starch that had been hydrolyzed with sulfuric acid which was heavily contaminated with arsenic. Analysis of the beer revealed that a gallon of beer contained anywhere from 65 to 195 mg of arsenic (Collins 1918).

Burning CCA-treated wood can also lead to unintentional exposures, such as, dermal exposure to soot and ash, and indoor air pollution. This was documented in a family who used scraps of CCA-treated plywood to heat their home during the winter. All eight members of the family experienced arsenic-associated health problems including pruritic dermatitis, pneumonia, severe diarrhea, peripheral neuropathy, hair loss, and reddening and thickening of the palms and soles (Peters et al. 1984).

Burning coal in homes can also lead to unintentional exposure. In the Guizhou Province of China, coal, which is traditionally burned in open pits inside the home for heat and cooking purposes, can contain up to 35,000 ppm of arsenic, and can produce arsenic concentrations in indoor air up to 400 mg/m^3 resulting in inhalation exposure and also contamination of food supplies stored in the home or cured over the coal fires (Liu et al. 2002).

95.3 ABSORPTION, METABOLISM, AND MECHANISMS OF TOXICITY

Approximately 80–90% of a single oral dose of inorganic arsenite (As_3) or inorganic arsenate (As_5) is absorbed from the gastrointestinal tract (Pomroy et al. 1980; Vahter and

Norin 1980; Freeman et al. 1995). Airborne arsenic can be readily absorbed through the lung, although the bioavailability and subsequent toxicity will likely depend upon the physicochemical properties of the arsenic compound (Jakubowski et al. 1998).

Percutaneous absorption of soluble arsenic compounds is also possible; however, dermal absorption rates are estimated to be 2.0–6.4% of the applied dose (Lowney 2005).

Once absorbed, arsenic is metabolized through a series of oxidative, reduction, and methylation reactions. This process begins when arsenate is reduced to arsenite in the bloodstream with glutathione (GSH) acting as an electron donor. *S*-Adenosyl methionine (SAM) then transfers a methyl group to arsenite that is subsequently oxidized to form monomethylarsonic acid (MMA_5). MMA_5 can undergo an additional reduction to form methylarsonous acid (MMA_3) that is methylated to form dimethylarsinic acid (DMA_5), which can be further reduced to dimethylarsinous acid (DMA_3) (Vahter 1999). All these species have been detected in urine collected from arsenic-exposed individuals (Thomas et al. 2004; Le et al. 2000).

Unlike other mammals, the average distribution of urinary arsenic concentrations in humans is 10–30% inorganic As, 10–20% MMA, and 60–70% DMA (Vahter 1999, 2000). Traditionally, methylation of inorganic arsenic has been considered a detoxification process because the mono- and dimethyl arsenic species are more readily excreted in urine than inorganic arsenic (Vahter and Marafante 1987). However, experimental studies indicate that the trivalent methylated arsenic intermediates (MMA_3 and DMA_3) may be more toxic than arsenite (As_3) or any of the pentavalent arsenic species (Ahmad et al. 1999; Nesnow et al. 2002; Styblo et al. 2002). Thus, factors that influence arsenic metabolism such as age, gender, nutritional status, or genetics could explain why some individuals are more susceptible to arsenic toxicity than others.

Arsenic toxicity is a product of the concentration and duration of exposure of each arsenic species at the target site. All organ systems are susceptible to arsenic. The mechanism of arsenic toxicity is poorly understood. At a biochemical level, pentavalent arsenic can replace phosphate in chemical reactions and trivalent arsenic has a high affinity for thiol groups, which can disrupt cellular enzymes and uncouple oxidative phosphorylation (ATSDR 1990). In addition, trivalent arsenic species generate reactive oxygen species, which could explain arsenic's carcinogenic properties. For instance, DMA_3 reacts with molecular oxygen to form a dimethylarsinic radical and a superoxide anion. This superoxide anion then generates hydrogen peroxide which can form a hydroxyl radical that can damage deoxyribonucleic acid (DNA) (Hei et al. 1998; Liu et al. 2001).

95.4 DERMAL EFFECTS

There is sufficient human evidence to classify arsenic as a known human carcinogen even though experiments in animal models are somewhat inconclusive (IARC 2004). The first report of arsenic-induced skin cancer was published in 1885 by Dr. White of Harvard Medical School, who described ulcerative lesions on the palms in a patient who took Fowler's solution for a number of years to treat psoriasis; the lesions appeared to be epitheliomas (White 1885). It was Hutchinson who consulted Dr. White's patient later in England and first suggested that arsenic could be a cause of skin cancer (Hutchinson 1888). The carcinogenic effect of Fowler's solution was later proven in rats whose skins were brushed with a 1.8% solution of potassium arsenite (Leitch and Kennaway 1922). Epidemiological studies have demonstrated that the odds of developing skin cancer including Bowen's disease, squamous cell carcinoma, and basal cell carcinoma are significantly associated with drinking arsenic-contaminated water (Tseng et al. 1986).

Skin is a target site for arsenic toxicity because trivalent arsenic has a high affinity for sulfhydryl groups, which are highly concentrated in keratin (Yu et al. 2006). In human skin cell lines comprised of keratinocytes, melanocytes, or dendritic cells, arsenic demonstrated both cytotoxic and genotoxic activities (Graham-Evans et al. 2004). Although the mode of arsenic carcinogenicity has not been fully established, it is thought that arsenite (As_3) binds to thiol groups and inhibits DNA repair whereas arsenate (As_5) replaces phosphates in DNA causing chromosomal aberrations and deletion mutations. Arsenic also alters DNA methylation and suppresses keratinocyte differentiation (Yu et al. 2006).

Chronic arsenic exposure induces a series of characteristic skin changes proceeding from hyperpigmentation to hyperkeratosis (Rahman et al. 2001). Significant associations have been observed between hyperpigmentation and palmar/plantar hyperkeratosis and risk of skin cancers (Yu et al. 2006). A unique characteristic of arsenic-induced Bowen's disease (carcinoma *in situ*) is that they are confined to sun-protected regions of the body, unlike sun-induced Bowen's disease. This difference could be explained by an interaction between arsenic and ultraviolet (UV) light. *In vitro* experiments have observed that combined exposure to UVB irradiation and arsenic increases the number of apoptotic cells resulting in an inhibitory effect on cellular proliferation (Lee et al. 2004; Yu et al. 2006).

95.5 BIOMARKERS OF ARSENIC EXPOSURE

Biomarkers are useful tools for evaluating exposure to environmental pollutants as they are quantitative measures of biologically relevant doses and reflect the internal dose from all exposure pathways. Arsenic can be measured in the blood, hair, nails, and urine, although blood arsenic concentrations are considered a poor biomarker of exposure because arsenic is cleared rapidly from the bloodstream and excreted via the kidney.

In urine, a single dose of ingested arsenic has a half-life of approximately 26 h (Buchet et al. 1981a), whereas the half-life of repeated oral exposures will be approximately 3 days (Buchet et al. 1981b), making urinary arsenic measures a useful biomarker for recent exposures. Background urinary

arsenic concentrations for unexposed individuals range from 0.1 to 1.0 ppm. It is important to realize that urinary arsenic measurements can be confounded by seafood consumption (Murer et al. 1992; Buchet et al. 1994). Therefore, if urinary arsenic is being used to determine exposure status, the patient should not eat fish for 3 days prior to the collection of the urine sample if total arsenic is being measured. However, if an analytical technique is being used that can speciate arsenic metabolites, then it is possible to remove the interference from the organic arsenic contributed by the seafood.

Hair and toenails, on the other hand, are useful biomarkers for historical exposures. Once inorganic arsenic is bound to keratin, it is isolated from metabolic activity and will subsequently accumulate with repeated exposures (Kile et al. 2005). Average background arsenic concentration in nails ranges from 0.43 to 1.08 ppm, whereas median background arsenic concentration in hair is approximately 0.5 ppm (NAS 1977). Furthermore, hair and nails can be collected noninvasively and do not require specialized handling or storage. Of these keratin-based biomarkers, toenails are better protected from external environmental contaminants and chemical treatments making them an ideal biomarker. However, care should be taken to remove any external contamination.

95.6 ARSENIC-INDUCED SKIN PIGMENTATION

The earliest symptoms of chronic arsenic toxicity are pigmentation changes in the skin and the thickening of the outer horny layer of the palms and soles. Generally, pigmentation changes occur first and can include a finely freckled pattern of hyperpigmentation and hypopigmentation of the skin on upper chest, arms, and legs, poetically described as "raindrops on a dusty road." More specifically, this raindrop pattern consists of less pigmented, round spots that are several millimeters in diameter on a background of diffuse hyperpigmentation (Figures 95.1 and 95.2).

The early stages of keratosis are characterized by bilateral thickening of the palms and soles. Subsequently, multiple nontender, horny papules develop on the keratotic skin of the palms and soles, although they may also develop on the dorsum of the hands (Figure 95.3). The papules are small, ranging from 0.2 to 1 cm in diameter, which can coalesce to form larger plaques with nodular, wart-like, or horny appearance. According to Mazumder (2003), chronic arsenic toxicity can be diagnosed from observing the presence of hyperpigmentation, keratosis, or both.

Traditionally, hyperpigmentation was linked to both arsenic deposition in melanocytes and increased melanin production (Granstein and Sober 1981), although recent studies have found underlying toxic effect of arsenic on both melanocytes and keratinocytes. The toxic effects on keratinocytes would be relevant to hyperkeratosis (Graham-Evans et al. 2004).

Chronic arsenic exposure has been associated with an increased risk of developing basal and squamous cell skin cancer (Tseng et al. 1986; Alain et al. 1993; Yu et al. 2006), and hyperpigmentation and keratosis are considered to be premalignant conditions.

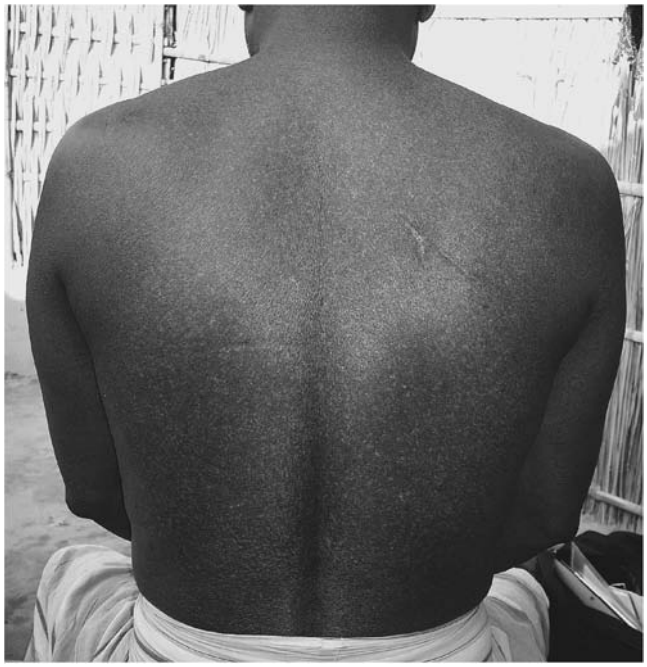


FIGURE 95.1 Characteristic raindrop pattern of hyperpigmentation associated with ingestion of arsenic-contaminated water (Pabna, Bangladesh).

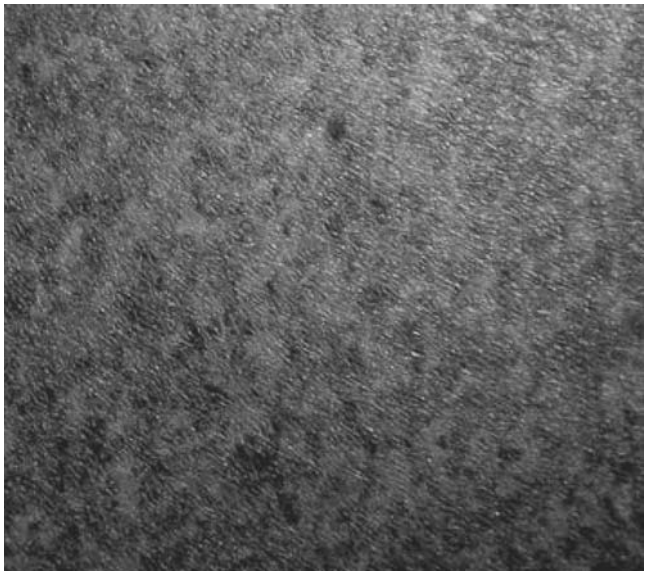


FIGURE 95.2 Close-up of hyperpigmented skin area (corresponds to approximately 3 cm × 3 cm skin area).



FIGURE 95.3 Plantar hyperkeratosis and pigmentation (Pabna, Bangladesh).

95.7 EPIDEMIOLOGICAL EVIDENCE OF ARSENIC-INDUCED SKIN PIGMENTATION

In West Bengal, India, and Bangladesh, millions of individuals have been exposed to arsenic-contaminated drinking water from shallow tube wells. In these populations, it has been reported that arsenic-induced skin lesions appear after 5–10 years of exposure to arsenic-contaminated drinking water (Mazumder et al. 1998b). However, arsenic-induced hyperpigmentation has been observed in children as young as 18 months in Bangladesh. The type of skin lesion and the severity of the lesion likely depend on both the ingested dose and the duration of exposure.

A population-based survey conducted in Matlab, Bangladesh, which screened 166,934 individuals above 4 years of age for arsenic-induced skin lesions, observed a crude prevalence of 0.3% (Rahman et al. 2006). This study found that 39% of the identified cases only had pigmentation changes, 5% only had keratosis, and the remaining 56% had both types of skin lesions. They also observed that men had a higher prevalence of skin lesions than women, which has also been reported by other researchers. (Mazumder et al. 1998b; Tondel et al. 1999; Watanabe et al. 2001; Hadi and Parveen 2004).

The prevalence of skin lesions is related to arsenic exposure. Age-adjusted prevalence rate for skin lesions in males ranged from 18.6 per 100 skin lesion cases when drinking water arsenic concentrations were below 150 μg/L to 37.0 per 100 when drinking water arsenic concentrations were above 1000 μg/L (Tondel et al. 1999). The prevalence of each type of skin lesions also appears to be influenced by exposure. Mazumder et al. (1998b) observed that the age-adjusted prevalence of keratosis ranged from 0.95 per 100 for drinking water arsenic concentrations ranging from 50 to 99 μg/L up to 9.5 per 100 when drinking water arsenic concentrations were above 800 μg/L. On the other hand, age-adjusted prevalence rates of hyperpigmentation ranged from 2.0 per 100 for drinking water arsenic concentrations ranging from 50 to 99 μg/L up to 17.1 per 100 when drinking water arsenic concentrations were above 800 μg/L.

Epidemiological studies in other populations exposed to arsenic-contaminated drinking water report different prevalence rates of arsenic-induced skin lesions. In Taiwan, the prevalence of hyperpigmentation and hyperkeratosis was 18.3 and 7.1%, respectively (Tseng 1968), whereas a survey of arsenicosis patients in North Mexico found that 12% of the patients had skin hyperpigmentation, 18% had hypopigmentation, and 11% had hyperkeratosis (Cebrian et al. 1983). However, as the detailed description of the observed lesions is omitted, the high prevalence of "hypopigmentation" could merely reflect terminology if the authors meant the hypopigmented spots on hyperpigmented background instead of "raindrop hyperpigmentation."

It is difficult to compare the findings of these different studies because it is possible that these different prevalence rates are a reflection of the study inclusion criteria. Currently, there are no widely accepted diagnostic criteria of arsenicosis, which could have a profound influence on epidemiological findings (Centeno et al. 2002). Also, many studies rely upon a field diagnosis of skin lesions, which could lead to misclassification particularly if the skin lesion is moderate or no exposure assessment is completed.

In cases of acute or subacute arsenic intoxications in European countries, the occurrence of hyperkeratosis, not hyperpigmentation, is more frequently reported. This was observed in a cohort of German wine growers, who would drink a wine substitute made from pressed grapes called "Haustrunk" that had been contaminated with arsenic-based pesticides (Luchtrath 1983). Among the 163 wine growers, who consumed an estimated 3–30 mg arsenic from Haustrunk daily, 77% presented with hyperkeratosis and only 44% had hyperpigmentation.

A large meta-analysis of 143 cases of skin cancer due to arsenic exposure performed by Neubauer (1947) found reports of hyperkeratosis in 116 cases (81%), whereas hyperpigmentation was only mentioned in 25 cases (17%) although direct information on the absence of hyperpigmentation was only provided for 11 cases, leaving room for speculations about the real hyperpigmentation prevalence.

Fierz (1965) mentioned that among 262 cases of side effects of skin diseases treatment with inorganic arsenic, hyperkeratosis was the most frequent (40.4%), whereas hyperpigmentation was found only in five patients.

These reports on the prevalence of different types of arsenic-induced skin lesions give a general impression that in European countries the prevalence of hyperpigmentation is lower than that in Asian countries and that keratosis might be the most frequently encountered indication of arsenicosis in European populations. It is unclear what would cause a higher prevalence of keratosis in Europe and Mexico compared to Asian countries, although genetic differences in arsenic metabolism, racial specificity of melanocytes function, or confounding from sun exposure could be involved.

95.8 DOSE–RESPONSE RELATIONSHIP

There is much uncertainty regarding the dose–response relationship between arsenic and skin lesions. This is largely due to the magnification of arsenic toxicity by dose and duration of exposure and the different methodologies employed in epidemiological studies.

For instance, a study conducted in Mexico specified that a person must consume approximately 2 g of arsenic before developing hypopigmentation and 3 g of arsenic before developing hyperpigmentation and hyperkeratosis for 8–12 years, respectively (Cebrian et al. 1983).

A study in Bangladesh found that the odds of arsenic-induced skin lesions and arsenic-contaminated drinking water increased in a dose-dependent fashion. For instance, it was observed that individuals drinking water containing 8.1–40.0, 40.1–91.0, 91.1–175.0, and 175.1–864.0 μg As/L had adjusted prevalence odds ratios of skin lesions of 1.91, 1.26, 3.03, and 3.71, respectively, and were 5.39 times more likely to have arsenic-induced skin lesions compared to individuals drinking water with less than 8.1 μg As/L (Ahsan

et al. 2006). This study is also one of the first to estimate the effect of chronic low-level arsenic exposure on the risk of developing skin lesions. Specifically, they observed that individuals consuming drinking water with 10 µg As/L had a 1.22 times higher risk of developing skin lesions compared to individuals drinking arsenic-free water. However, the relationship between drinking water arsenic concentrations and the type of skin lesion was not significant even though hyperpigmentation is considered to be an earlier sign of arsenicosis compared to hyperkeratosis (Ahsan et al. 2006).

95.9 TREATMENT

Despite mankind's long history with arsenic, no treatment currently exists that can reverse chronic arsenic toxicity. Therefore, all efforts should be made to identify and eliminate the source of arsenic exposure.

In acute poisoning, chelation therapy is frequently employed to facilitate excretion of arsenic in urine. In the United States, two chelating agents are available: 2,3-dimercapto-1-propanol (dimercaprol or British Anti-Lewsite [BAL]) and *meso*-2,3-dimercaptosuccinic acid (Succimer). A third chelating agent, sodium 2,3-dimercapto-1-propane sulfonate (DMPS), is available in Germany. BAL is administered through intramuscular injections and has a narrow therapeutic:toxic ratio and a high rate of side effects. Succimer and DMPS, on the other hand, are given orally or intravenously and are more widely tolerated. All three chelating agents increase the urinary excretion of arsenic, although no controlled studies have been performed to determine the most effectual therapy.

In cases of chronic arsenic exposure, it is unclear whether chelation therapy offers any benefit. One small placebo-controlled study found that patients given DMPS four times a day for 1 week, and repeated in the third, fifth, and seventh week, had significantly increased urinary excretion of arsenic compared to the patients given a placebo. Both groups showed significant improvement in their clinical conditions, although the DMPS group had more improvement (Mazumder et al. 2001). The clinical improvement in the placebo group was attributed to nutritious diet and the cessation of arsenic exposure. An earlier study using Succimer as the chelating agent did not observe any difference in the clinical improvement compared to the placebo group (Mazumder et al. 1998a). Therefore, it appears that DMPS is a superior chelation therapy for chronic arsenicosis, although providing clean drinking water and a good diet are also effective treatments for chronic arsenicosis.

It is believed that antioxidants, such as vitamin E and selenium, may impede carcinogenic effects of arsenic. It was shown that supplementation with vitamin E and organoselenium, either alone or in combination, slightly improved the status of arsenic-induced skin lesion, although the observed improvement was similar to the improvement observed in the placebo group (Verret et al. 2005). This finding supports earlier research that suggests that for chronic arsenicosis, removing the source of arsenic exposure and eating a well-balanced diet is important.

95.10 CONCLUSIONS

In Asian countries, changes in skin pigmentation are the first sign of chronic arsenic toxicity. This can include diffuse hyperpigmentation with scattered hypopigmentation. This condition can occur prior to, or simultaneously with, keratosis of the palms and soles. In European countries, keratosis might be the first indication of chronic arsenic toxicity. Considering that these arsenic-induced skin lesions are earliest signs of arsenic poisoning, and also a frequent predecessor of skin cancer, it is important to diagnose these skin lesions as early as possible to identify populations at risk.

In addition, collaborative efforts should be undertaken to develop an international consensus on diagnosis of arsenical hyperpigmentation. Changes in skin pigmentation can be objectively documented with digital cameras and also quantitatively measured with portable colorimetric devices, which would help to standardize field examinations of the patients with risk of arsenicosis. The same technique could be used to determine changes in lesions as a result of treatment. Needless to say, the precise measurement of pigmentation changes can also be used to obtain more precise information in epidemiological studies, which may additionally contribute to better understanding of arsenic toxicity mechanisms that are not fully understood.

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96 Textiles and Human Skin, Microclimate, Cutaneous Reactions: Overview

Wen Zhong, Malcolm M.Q. Xing, Ning Pan, and Howard I. Maibach

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96.1 INTRODUCTION

The skin is a large barrier organ that protects the human body from environmental hazards (heat, cold, chemicals, mechanical forces, etc.), and maintains the integrity of the body, while the clothing system provides an extra layer(s) of barrier to enhance the esthetic, thermophysiological, and sensorial comfort of the wearer. However, direct contact and interactions between textiles and skin may cause reactions, even damage or diseases.

This chapter overviews research in the interdisciplinary area of textile/skin interaction and related skin reactions and injuries. First, a brief description relates microclimate in the skin/clothing system and especially the skin responses to moisture and heat transfer within this system, as this plays a critical role in skin irritation and intolerance caused by textiles. Then follows a discussion on skin irritation reactions to textiles, including dermatitis caused by chemicals (dyes and finishes) and physical contact/friction. Finally, two skin injuries—blisters and pressure ulcers—which are caused by physical contact, pressure, and friction, are discussed. And the role that textiles play in the prevention and formation of these injuries are examined.

96.2 MICROCLIMATE

The stratum corneum (SC) plays an important role in the clinical appearance of the skin as a result of its water-holding capacity and lipid content [1,2]. From the deeper, highly hydrated layers of the epidermis and dermis, a passive flux of water takes place toward the more superficial SC layers, which have a relatively low water content. This is the so-called transepidermal water loss (TEWL) [3], which is a parameter to evaluate the function of SC as a barrier to prevent excessive water loss.

Extensive research work has been published on the topic of TEWL [4–7]; however, knowledge about the influence of textile materials on TEWL is limited.

In 1987, Hatch et al. reported an *in vivo* study of water content in the surface layers of human SC and water evaporation from its surface due to placement of fabric on skin for varying time periods [8]. A lightweight fabric placed on skin produced no change in skin water content or evaporative water loss from the SC. Only for occluded treatments (e.g., fabric plus plastic film) did water content and evaporation increase as the covering materials remained for longer periods.

Another water-loss route through skin is via perspiration or sweat, which is secreted by eccrine sweat glands deep in dermis. Water evaporation from the secretion absorbs heat, and thus helps regulate body temperature in response to environmental changes. For humans to feel comfortable, a fairly narrow surface temperature and humidity must be maintained in the air immediately surrounding the body. Clothing, therefore, plays an important role in regulating body temperature and controlling heat loss. The term microclimate, accordingly, has been used frequently to describe the environmental parameters that influence heat exchanges such as the temperature, humidity, and microspace air stream between the skin and clothing [9]. Microclimate is an important factor for wear comfort, and depends on properties such as moisture

and heat transport through the material, and on physiological and environmental conditions.

Clothing comfort has been extensively studied; however, less has been done on the skin response to fabric in various conditions. Hatch and colleagues published on *in vivo* cutaneous and perceived comfort response to fabric [10–15]; this series began with experiments in a simulated skin/fabric/microclimate system, which is composed of a modified Kawabata Thermolabo apparatus housed in a controlled environmental chamber [12]. The three experimental fabrics (one cotton and two polyester fabrics with different fiber deniers) showed small differences in water vapor and air permeability as well as energy dissipation rates. The results suggested that these thermophysiological comfort parameters *related more to fabric structures than to fiber contents*. In addition, different mechanical and surface properties of fibers may contribute to variation in sensorial comfort of the experimental fabrics [10]. They then documented water content and blood flow in human skin under garments worn by exercising subjects in a hot and humid environment [11]; no significant differences were observed between the three experimental fabrics in terms of alteration in capillary blood flow, SC water content, skin evaporative water loss, or skin temperature [13]. Surprised by the results, further investigations were performed when fabric patches were placed directly in contact with volar forearm skin of subjects instead of clothing worn loosely by the subjects [14,15]. The experiments revealed that the SC hydration reduced after being in contact even with hydrophilic fiber (cotton).

Kwon et al. compared the physiological effects of the hydrophilic and hydrophobic properties of the fabrics in exercising and resting subjects with and without wind [16]. Materials included three kinds of clothing ensemble with different moisture regains (wool/cotton blend with high moisture regain; 100% cotton with intermediate regain; 100% polyester with low regain). They concluded that the hydrophilic properties of the fabrics studied were of physiological significance for reducing heat strain, including skin temperature, clothing microclimate temperature and humidity, and pulse rate, during both exercise and rest especially when influenced by wind.

Generally, the experiments and analysis on the skin response to textile and clothing system have yet to lead to commercial interventions. This may be caused by the individual differences among human subjects in terms of physical status and sensitivity. When it comes to the *in vitro* experiments, the difficulties lie in how to realistically represent the whole skin/fabric/microclimate system.

96.3 SKIN REACTIONS TO TEXTILES

The skin's irritant reactions to textiles may be caused by chemicals or physical contact and friction.

96.3.1 Allergic Contact Dermatitis

Numerous chemicals may be incorporated into the textiles and clothing during the processes from fiber formation, spinning, fabric construction to dyeing and finishing. These chemicals, when in contact with human skin, may cause allergic contact dermatitis (ACD).

Hatch and Maibach [17] reviewed the occurrence of dermatological problems caused by consumer exposure to dyes on clothing. Thirty-one dyes, mainly dispersed with anthraquinone or azo structures, have been suggested to cause ACD. Subsequently, they reviewed the literature concerning textile dye dermatitis published during the decade before, and four new dye allergens were identified [18]. Studies on ACD prevalence, the amount of ACD cases that are presented in a population, were summarized in 2000 [19]. Most studies, however, were conducted in Europe, primarily in Italy. And all the tests were performed by placing a dye, mostly disperse dye, with unknown purity instead of a dyed fabric directly on the skin.

Accordingly, they adopted the term "textile-dye ACD" in contrast to "color-textile ACD" [20], as the latter case involves more complicated factors like dye molecules transferred or released from textiles to the skin—perspiration fastness of the dyes. It was also reported that *dyes to which a patient was patch test positive were infrequently identified in the fabric suspected to be the cause of the skin problems* [21]. This means that further investigation is desired in the diagnosis and management of colored-textile ACD.

They further reviewed textile chemical finish dermatitis [22]. Chemicals used on fabrics to improve 10 performance characteristics have been detected to have resulted in irritant or ACD. The most significant problem is due to formaldehyde and *N*-methylol compounds for durable press fabrics. An updated review on textile dermatitis caused by resins, additives, and fibers ended in 1994 [23]. Textile formaldehyde resins for durable press finish were still the focus, as formaldehyde released from the resin was believed to be the causal agent.

Hatch and Maibach provided a list of textile chemicals (dyes, finishes, and additives) reported to cause textile dermatitis and the types of fabrics on which these chemicals are most likely to present [24]. Clinical aspects of textile dermatitis and methods available to identify the specific chemical causing a skin problem are also covered.

However, the extent of the skin problems caused by textile-associated chemicals is hard to define and predict, due to a series of factors including variation of skin's sensitivity, capacity of absorption and reaction among different people, transfer of irritant chemicals from textiles to skin, synergy of sweating, pressure and friction, etc.

96.3.2 Skin Irritation by Physical Contact/Friction

The frictional properties of skin are of interest in the area of cosmetic products and to clinical dermatologists dealing with acute and chronic friction trauma, such as blister and callus.

In 1990, a study on frictional properties of human forearm and vulvar skin was reported [25]. The dynamic friction coefficient between skin and a Teflon probe was measured in vulvar and forearm skin of 44 healthy female volunteers and its correlation with age, body weight, height, TEWL,

and skin capacitance was obtained using multiple regression analysis. They observed that a higher friction coefficient of vulvar skin (0.66 ± 0.03) than that of forearm (0.48 ± 0.01) may be due to the increased hydration of vulvar skin. And age differences seem to exist for TEWL and friction coefficient in forearm but not in vulvar skin.

A similar study on skin friction properties involved human subjects of different gender and age [26]. Measurements were obtained from 11 anatomical regions, namely the forehead, upper arm, volar and dorsal forearm, postauricular, palm, abdomen, upper and lower back, thigh, and ankle. The dynamic friction coefficient did not vary significantly between age and sex groups but varied considerably among the anatomical regions. They suggested that frictional properties of skin are dependent more on water content or nonapparent sweating and the role of sebum secretion is suggested as one possible factor. And a later study suggested that the surface lipid content (SSL) plays a limited role in frictional properties of skin [27].

Other studies on the influence of skin friction on the perception of fabric texture and pleasantness under a series of environmental conditions from neutral to hot-dry and hot-humid also revealed that moisture on the skin surface increased skin friction [28] and that fiber type and moisture influenced fabric-to-skin friction measurements [29]. These reports agreed that *moisture on the skin is more important than the fiber type or fabric construction parameters* in determining the nature and intensity of fabric-to-skin friction and that *glabrous skin friction changes less with wetting than with hairy skin*.

Recent studies have further investigated the role of moisture, sebum, and emollient products on skin friction properties [30]. Elkhyat et al. recorded the influence of hydrophilic/hydrophobic balance (Hi/Ho) of the skin surface on the friction coefficient, using both *in vitro* and *in vivo* experiments [31]. They showed that the higher the hydrophobia tendency of the surfaces, the lower the friction coefficient. The friction coefficient, therefore, may quantify the influence of lubrificant/ emolients/ moisturizers applied to the skin. And the relationship between the friction coefficient and the hydrophilic/hydrophobic balance can be reversed in the presence of water and sebum on forehead.

With regard to the experimental methods for measuring frictional coefficient of the skin, the earlier designs fall into two categories: using either a probe moved across the skin in a linear fashion [32] or a rotating probe in contact with the skin surface [33,34], as also described in a review article [35]. Recently, there are reports about instruments capable of measuring friction coefficient of skin in real time, such as a commercially available universal micro-tribometer (UMT) series Micro-Tribometer. Either a stainless steel ball [36] or a copper cylindrical friction/electrical probe [37] was pressed on to the skin with a preset load and moved across the skin at a constantly low velocity. The UMT continuously monitored the frictional force of the skin and the normal force applied by the probe to calculate the friction coefficient in real time. Another commercial device for measuring surface properties of textile materials, a KES-SE Frictional Analyzer [38], was used in skin friction evaluation [39], where the Average coefficient of friction (MIU) and its Mean deviation of MIU (MMD) were used as parameters to indicate surface friction. In addition, Tanaka et al. launched a study on a device for monitoring skin conditions, including roughness and softness [40]. The device, so-called "Haptic Finger," was designed using Polyvinylidene fluoride (PVDF) piezopolymer film as a sensory receptor. Signals obtained by sliding the sensor over skin surfaces were processed by wavelet analysis, and the dispersion of the power spectrum density in the frequency domain was obtained and found to be associated with roughness and hardness of skin in both *in vitro* and *in vivo* experiments.

However, measurements of the friction coefficient of skin by different devices lack comparability, for there is still disagreement on which scientific law governs the relationship between the pressure and skin friction. The classic Amonton's law [41], which stipulates that the friction coefficient remains unchanged under varying normal loads and speeds of the probe (i.e., the opposing material used to measure the skin friction), was long challenged by numerous research works including some recent ones [36,39], in which the friction coefficient is found to be inversely proportional to load [42].

Compared to what was achieved in measuring frictional coefficient of skin surface, far less work was performed in the assessment of frictional force between skin and fabric. This usually involved slowly pulling fabric samples across the surface of a subject's skin (i.e., forearm). The frictional force required to pull each fabric across the skin was recorded by a force transducer. The pressure between fabric and skin was often applied by suspending a weight to the free end of the fabric. The resulting irritation effects caused by friction could then be documented [28,29]. Other methods for measuring skin/fabric frictions were achieved by using strain gauge [43] or strained gauged flexure couples which are arranged in a way trying to detect both normal and frictional forces [44]. Measurements can be made when wiping the material with the right index finger.

Literature concentrating on the skin irritation caused by contact and friction of clothing or other textile materials has been summarized by Hatch and Maibach [45]. Six fibers that had been reported to be linked to dermatological problems were covered: nylon, for contact dermatitis and contact urticaria; wool, for acute and cumulative irritant dermatitis, aggravate atopic dermatitis, ACD, and immulogic contact urticaria; silk, to atopic dermatitis and contact urticaria; glass fiber, to mechanical irritation; and spandex and rubber fibers. Some dermatological problems, such as in the cases of nylon, spandex, and rubber fibers, were often caused by dye, finish, or fiber additive instead of fiber material itself.

A study on the effects of wearing diapers on skin showed that skin wetness was proportional to diaper wetness, and, with increased skin wetness, there were increased coefficients of friction and increased abrasion damage [46]. Studying the electrostatic potentials generated on the surface of the scrotal area, the accumulated electrostatic charges on the pants were due to the friction of the pants with the skin, when different types of textile fabric were worn [47]. The polyester

pants showed the highest potential while the polycotton pants produced less than half that level. The readings at daytime were higher than at night, probably due mainly to the higher temperature and activities during the day. A related study even suggested that this electrostatic potential may be responsible for inhibiting hair growth [48].

In an effort to develop test methods to evaluate certain consumer products, such as feminine hygiene products and diapers, for their potential in causing mechanical irritation during use, Farage et al. investigated several test sites on the human body where normal daily activities provided the opportunity for movement and therefore friction [49]. These studies indicate that a protocol using the back of the knee as a test site with an exposure regimen of 6 h daily for 4 days proved to be the most effective test system for evaluating mechanical irritation.

Prolonged or extensive contact combined by pressure, friction, or shear between fabric and skin may lead to more serious problems or injuries, such as friction blisters and pressure ulcers, as discussed in the next section.

96.4 SKIN INJURIES

96.4.1 Friction Blisters

Friction blister is a frequently occuring skin problem associated with sports and vigorous activities. It can be critical if they occur during athletic competitions or military missions, when reduced performance or mobility becomes costly, injurious, or fatal. Accordingly, extensive research has been performed on the blister, causing fabric/skin friction and interactions.

Studies showed that blisters result from frictional forces that mechanically separate epidermal cells at the level of the stratum spinosum. Hydrostatic pressure causes the area of separation to fill with a fluid that is similar in composition to plasma but has a lower protein level [50].

There were a series of reports on a specially designed apparatus for producing friction blisters on human skin in late 1960s and early 1970s [51–55]. The instrument consisted of a rubbing head to which various materials (including textiles) could be firmly attached. The head could be moved over the surface of any chosen skin site at a selected stroking rate under a known amount of load. Frictional coefficient and temperature could also be recorded. Observations [54] and healing treatments [52] were performed on blisters formed by the instrument on human volunteers.

The other studies on friction blister (mostly foot blisters) formation and prevention were usually performed by recording the prevalence and size of blisters among a group of subjects with routinely heavy load of activities, such as athletes or military personnel [56–59].

For example, Herring and Richie conducted a double-blind study to determine the effect of sock fiber composition on the frequency and size of blistering events in long-distance runners [57]. Socks were tested, which were identical in every aspect of construction except fiber composition: One was composed of 100% acrylic and the other 100% natural cotton fibers. Acrylic fiber socks were associated with fewer blistering events and smaller blister compared to cotton fiber socks.

Another examination into the effect of sock fiber contents on the incidence and severity of foot blister was reported by Knapik et al. [59]. Three hundred and fifty-seven military trainees were divided into three groups and assigned one of the three boot-sock system: the standard military boot sock consisting of a wool–cotton–nylon–Spandex combination; the standard military boot sock with a thin inner or liner sock consisting of polyester; a very thick, dense, prototype outer sock consisting of a wool–polypropylene combination over the same liner sock as the second group. The standard military sock with a polyester liner reduced the incidence of severe blisters, but the dense sock with the polyester liner reduced the overall incidence of blisters as well as the incidence of severe blisters.

Patterson et al. studied the blister attach rate among 100 cadets in a summer camp [58]. Studies showed that women had higher risk than men. Cadets with a history of blisters in the 2 years before camp had an increased relative risk of blister formation. It was also suggested that the foot should be preconditioned to its footwear to prevent blister formation, say wearing the boots over 20 h/week during the 2 weeks immediately before camp.

Other measures to prevent blister formation include lubrication [60], decreasing friction/shear [61], or reducing the skin surface hydration as moist skin increases frictional force [50]. However, very dry or very wet skin would decrease frictional forces.

Reynolds et al. investigated the influence of an antiperspirant with emollient additives on frequency and severity of frictional blisters, hot spots, and irritant dermatitis by having 23 healthy subjects walking on a treadmill in a warm environment [62]. However, the results showed that it reduces irritant dermatitis but does not reduce foot-sweat accumulation, blister or hot spot incidence, or blister severity. A later study was carried out on the effect of an antiperspirant in reducing foot blisters during hiking [63]; it might be effective in reducing foot blisters during hiking; however, a side effect of skin irritation was observed.

Despite extensive studies on friction blisters, the prevalence or severity of friction blister is still difficult to predict, let alone a simple solution to prevent its formation. The cause may lie in the dramatic variation of skin conditions (surface roughness, hydration, adhesion between skin layers, etc.) among individuals as well as among different anatomic sites of the same person.

96.4.2 Pressure Ulcers

Pressure ulcer, defined as an area of localized damage to the skin and underlying tissue caused by pressure, shear, friction, or a combination of these [64], presents a significant healthcare threat to hospitalized patients. Approximately 1 million hospitalized and nursing home patients are diagnosed with pressure ulcers and about 60,000 die as a result of pressure ulcer complications annually [65]. Related costs have been estimated to exceed $1 billion annually in the United States [66,67].

According to etiology of pressure ulcer formation, when compressive and shear/friction forces reach certain threshold (combination of intensity and duration), there is occlusion and thrombosis of capillary at pressure points or areas. This results in tissue anoxia with release of toxic metabolites, and ultimately cell death and tissue necrosis. Pressure ulcers are thus formed [64,68].

As the principal mechanical factors intriguing ulcer formation, the combination of pressure and shear/friction has been reported to be devastating to the skin and underlying tissues. Dinsdale demonstrated that when both pressure and friction were applied to the skin of swine, a pressure as low as 45 mmHg was sufficient to cause an ulcer, while 290 mmHg of pressure was required if no friction was present [69]. Davis presented hypotheses of three scenarios with different shear and vertical force conditions that could lead to skin ulceration [70]: at a localized area the skin may tend to slip (1) toward, (2) away from, or (3) parallel to a neighboring skin region where the two skin regions possess different friction coefficients against the slippage.

Despite all the different scales for assessing pressure ulcers, there are some common factors that are included or considered [71,72], namely pressure, shear/friction, and liquid/moisture. Among the overwhelming publications in pressure ulcers research including updated reviews [59, 73–75], little has been devoted to the role that textiles play in the formation and prevention of pressure ulcer, although textiles could interfere with all the following important factors associated with pressure ulcers:

1. Pressure. Although the fabrics (clothing and beddings) alone cannot do much to reduce the pressure experienced by patients (other solutions like repositioning, using pillows/cushions/foam wedges, or using low-pressure mattress or seat that can better perform the job [76–78]), they would play a critical role in governing the shear and friction actions on human skin once pressure and body motion are involved.

   Nevertheless, there have been studies on specially designed clothing/socks in terms of their effectiveness in prevention and management of pressure ulcers. For example, padded hosiery has been reported to reduce plantar pressures in patients at risk of ulceration [79]. Specially designed socks, when worn with suitable shoes, may be an acceptable and inexpensive addition to existing methods of protecting the high-risk insensitive diabetic foot ulceration [80].
2. Shear stress and friction. The surface smoothness of fabrics and stiffness/flexibility of fiber and fabric may be two of the important factors in determining the shear and friction experienced by patients. Little has been done on the effort of shear/friction monitoring in preventing pressure ulcers. Snycerski and Frontczak-Wasiak presented a design and manufacture of a double-layer woven fabric for bedsheet with different friction coefficients on both sides of the fabric [81]: the bottom side of the bedsheet has a higher friction coefficient so as to limit the slip between the bedsheet and underlying bedding materials and therefore reduce bedsheet wrinkling; the up side of the bed sheet has a low friction coefficient to allow easier and smoother position change for patients. However, the efficacy of this sheet in controlling pressure ulcers has not been reported.
3. Liquid/moisture or skin hydration. Appropriate moisture conditions should be maintained to prevent or reduce ulceration. An overdry condition may lead to a skin more vulnerable to cracking. Conversely, a wet condition (because of incontinence and perspiration) may cause skin maceration and lower the tissue tolerance to shear stress and friction [68]. It may also create a favorable condition for the growth of microorganisms. The clothing (and the bedding) system plays an important role in moderating liquid and moisture so as to maintain a healthier microclimate near the skin surface.

The role played by textiles in the formation and prevention of pressure ulcers is generally understudied, despite that the textiles (clothing and bedding) could have considerable influence on the factors (pressure, shear/friction, and skin hydration) contributing to skin ulceration. More research effort, therefore, is expected in this field for a better understanding as well as a more efficient way in controlling the problem.

96.5 SUMMARY

Skin provides the critical first defense mechanism for the body in dealing with external hazards. Clothing fabrics and the skin surface constitute a buffering system that establishes a thermal and sensorial state of comfort to maintain human health and normal functions. A failure of fabric/skin regulatory interactions can lead to various problems, from thermophysiological discomfort, irritation, to injuries such as blisters and pressure ulcers.

We reviewed here the existing studies in the fabric/skin interactions, related irritation reactions, and injuries. The microclimate between clothing and skin surface, where fabric/skin interactions take place, has been discussed. Skin irritations caused by both textile chemicals and physical skin-textile contact/friction have also been reviewed. The final section deals with skin injuries, blisters, and pressure ulcers, caused by physical contact, pressure, and friction.

Despite the prevalent problems caused by ill textile/skin interactions, few research efforts have been devoted to this field. In addition, the existing *in vivo* experimental studies have rarely led to any significant results and solid conclusions. The cause may lie in the dramatic variation of skin conditions (surface roughness, hydration, adhesion between skin layers, etc.) among individuals as well as among different anatomic sites of the same person. Another reason might be the lack of communication between researchers in the areas of textiles and dermatology.

Literature Summary Table

Topic	Results	Ref.	Comments
Microclimate			
TEWL of human skin		1–7	Dermatological studies
Microclimate		9	Concept
In vivo cutaneous and perceived comfort response to fabrics			Individual differences among human subjects in terms of physical status and sensitivity. Has yet to lead to commercial interventions
Water content and evaporation on human skin surface	Light weight fabrics produced no changes	8	
Experiments in simulated skin/fabric/microclimate system	Thermophysiological comfort parameters related more to fabric structure than to fiber content	12	
Mechanical and surface properties of fibers on sensorial comfort		10	
SC water content, evaporation and capillary blood flow in hot and humid environment	No significant differences between experimental fabrics	11,13	
Patch test on volar forearm skin	SC hydration reduced when in contact with hydrophilic fiber	14,15	
Physiological effects of hydrophilic properties of fabrics	Hydrophilic properties of fabrics were of physiological significance for reducing heat strain when influenced by wind	16	
Allergic contact dermatitis (ACD)			
Textile dye dermatitis	Review on identified dyes that cause skin problem, prevalence	17–19	Extent of the problems is hard to define and predict, due to variation of skin's sensitivity, capacity of absorption and reaction, transfer of irritant chemicals from textiles to skin
"Textile-dye ACD" and "Color-textile ACD"	Dyes to which a patient was patch test positive were infrequently identified in the fabric suspected to be the cause of skin problem	20,21	
Chemical finish dermatitis	Review of identified chemicals that cause skin problem	22–24	
Skin irritation by physical contact/friction			
Frictional properties of human skin	Frictional properties varies among human anatomical regions	25–27	Prolonged or extensive contact combined by pressure, friction, or shear between fabric and skin may lead to more serious problems or injuries, such as friction blisters and pressure ulcers
Moisture on skin friction	Moisture on skin is more important than fiber type in determining frictional properties	28,29	
Moisture, sebum, and emollient on skin friction	The higher the hydrophobia tendency of the surfaces, the lower the friction coefficient	30,31	
Measuring skin friction		35	
Probe moved across skin in linear fashion		32	
Rotating probe		33,34	
UMT series Micro-Tribometer	Measuring friction coefficient in real time	37	
KES-SE Frictional Analyzer	Recording friction coefficient and its mean deviation	38,39	
"Haptic Finger"	A sensory receptor	40	
Skin friction versus load			
Classic Amonton's law	Friction coefficient is unchanged with varying normal loads	41	
Challenged by recent work	Friction coefficient is inversely proportional to load	36,39,42	
Assessment of frictional force between skin and fabric	Frictional force required to pull each fabric across the skin is recorded by a force transducer	28,29	
	Frictional force measured by strain gauge or strained gauged flexure couples	43,44	
Skin irritation caused by textile fiber contact/friction	Review of identified fibers that cause skin problem	45	

Literature Summary Table (Continued)

Topic	Results	Ref.	Comments
Skin irritation by diaper, feminine hygiene products	Increase in friction and abrasion with increase in skin wetness	46,49	
Electrostatic potentials generated by fabric/skin friction	Polyester pants showed highest electrostatic potential	47,48	
Friction blister			
Blister formation		50	Prevalence or severity of friction blister difficult to predict, let alone a simple solution for prevention. This cause may lie in the dramatic variation of skin conditions among individuals and among different anatomic sites of the same person
Special apparatus for producing friction blisters on human skin	Observations and treatments performed on blisters formed by the instrument on human skin	51–55	
Blisters, studies on prevalence		56–59	
Blister prevention:			
Design of socks	Acrylic socks associated with fewer blistering than cotton	57,59	
Lubrication		60	
Decreasing friction/shear		61	
Reducing skin surface hydration	Antiperspirant might be effective in reducing foot blister	50,62,63	
Pressure ulcers			
Pressure ulcer, prevalence, and cost		64–67	Role of textiles in the formation and prevention of pressure ulcers is generally understudied, despite that textiles (clothing and bedding) could have considerable influence on the factors (pressure, shear/friction, and skin hydration) contributing to ulceration
Pressure ulcer formation			
Etiology		64,68	
Hypothesis on shear and vertical force conditions		70	
Principal mechanical factors		71,72	
Pressure	Most important factor	76–78	
Shear/friction	The presence of shear significantly reduce the threshold of pressure that intriguing ulcer formation	69	
Skin hydration		68	
Ulcer prevention, design for textile materials			
Pressure reduce (socks)	Padded hosiery may reduce planar pressure in patients at risk of ulceration	79,80	
Friction control (bedding)		81	

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97 *In Vivo* Human Transfer of Topical Bioactive Drugs among Individuals: Estradiol*

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97.1 INTRODUCTION

Topical chemical application was thought of as that for local drug dermatological treatment, or perhaps as a cosmetic application. Now bioactive drugs are topically administered for systemic treatment such as hormonal replacement estrogen therapy (Jewelewicz, 1997). Zondek (1938) reported that if a hormone (estrone) ointment or hormone oil is rubbed into the shaven skin of the back of a castrated mouse the animal goes into estrus. He also observed that the topical dose required seven times as much hormone as with subcutaneous injection. The seven times difference is due to the topical dose not absorbed through the skin. Feldmann and Maibach (1969) showed estradiol human percutaneous absorption to be 10.6% for 24-h dose application. The remaining dose was washed off or lost.

Studies in percutaneous absorption have shown that a portion of the topical chemical, generally a few percent of the applied dose, became systemically available. This few percent dose absorbed was sufficient for certain bioactive drugs and led to transdermal drug delivery for systemic application. During the topical drug application period, generally 24 h, mass balance has shown that the majority of the topically applied dose was still on the skin (Wester and Maibach, 1992). In essence, an excess of drug is on the skin through the complete topical dosing period.

Johnson et al. (1983) showed that in 50 hospitalized patients given tetracycline ointments, creams, lotions, and tinctures containing a fluorescent marker, the topically applied medications did not remain confined to sites of initial application. Yerasi et al. (1997) reported an unusual case

Dr. Kade (Dr. Kade Pharmazeutische Fabrik GMBH, Berlin, Germany) provided the funds and instruction on preparation of the gel. Otherwise, sponsor had no role in study design, data collection, data analysis, data interpretation, or writing of the report.
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where a man died of fentanyl poisoning, yet it was his wife who had the fentanyl patches. Wolf et al. (1997) reported childhood poisoning involving transdermal nicotine patches. Lu and Fenske (1999) reported dermal transfer of pesticide residues from residential surfaces. Franklin and Geffner (2003) reported a case of pronounced virilization of a 2-year-old boy from a topical testosterone cream being used by his father. Vihtamäki et al. (2004) showed skin contamination by estradiol gel to be a remarkable source of error in plasma estradiol measurements. Topical estradiol and testosterone preparations are available as well as other topical bioactive preparations. This study examines human skin *in vivo* percutaneous absorption of 17 β-estradiol, including transfer between individuals through skin.

97.2 MATERIALS AND METHODS

97.2.1 Dose Formulation

[^{14}C]-estradiol, [4-^{14}C]-NEC-127 estradiol with a specific activity of 54.1 mCi/mmol was obtained from DuPont NEN (lot number 3188-151SP), and was kept at 0–4°C until used. Radiochemical purity of 98.5% was determined by HPLC and TLC.

The formulation was a gel (estrogel) prepared according to the manufacturer. An appropriate amount of [^{14}C]-estradiol was incorporated to be a 0.06% (w/w) dose formulation containing 5 mg [^{14}C] estradiol radioactivity per 8.5 g of gel (131 μCi/g). The radioactive estradiol was incorporated into the gel. Triplicate aliquots were assayed to determine uniformity of formulation. Use of radioactivity was necessary to distinguish between that estradiol [^{14}C-] which was absorbed through the skin and that estradiol which is a natural constituent of the human body.

97.2.2 Study Design

Group A was to determine topical absorption of [^{14}C]-estradiol under a protective condition during a 24 h exposure period. The protective nonocclusive cover placed on the dosed area and kept for 24 h was to protect the dosed site and exclude drug loss. After 24 h, the dosed site was washed with soap and water and the washing sample was retained for ^{14}C assay. The cover was assayed for possible residual chemical.

Group B was to determine topical absorption on [^{14}C]-estradiol under normal use conditions, which had no protective device during the 24 h exposure period.

Group C was to determine how much [^{14}C]-estradiol was transferred after contact with group D. One hour after topical dose, the dose area was rubbed and contacted by a group D volunteer who did not receive any dose.

To prevent [^{14}C]-estradiol transferring from the dosed site skin to other skin area via contaminated clothes, a Tyvek paper sleeve (Lab Safety Supply) was placed on each dosed forearm and kept in place for the 24 h dosing period for groups B, C, and D volunteers. After 24 h, the dosed site was washed with soap and water, and the washings retained for ^{14}C assay. The sleeve was assayed for possible residual chemical.

The experimental design and performance followed the Helsinki Guidelines (Ethical Principles for Medical Research Involving Human Subjects, see: www.wma.net) and was approved by the Committee on Human Research at the University of California, San Francisco. Human volunteers were recruited from the University of California, San Francisco and surrounding San Francisco Bay Area community. Each volunteer signed a consent form before the study. Eighteen healthy postmenopausal women aged 42–78 years were selected for groups A, B, and C. Six normal, healthy men or women aged 29–76 years were selected for group D. Each group consisted of six volunteers.

97.2.3 Dosing Procedure

97.2.3.1 Group A

A 50 cm^2 area (3.5 cm × 14.3 cm) was marked on the left and right ventral forearms of each volunteer. For each forearm the marked areas received a single topical application of total 0.17 g (22 μCi) [^{14}C]-estradiol gel formulation using a 0.1 ml Teflon-coated syringe (Hamilton Company, Reno, NV). The delivered dose was quantitated by weighing the microsyringe before and after dosing. This was done for all dosings. After topical applications, a nonocclusive solid plastic cover was secured over each dosed area of group A volunteers and kept in place by tape for 24 h. The cover was made by cutting a plastic cylinder (20 cm length × 5 cm diameter) in half lengthwise, and drilling three 1 cm diameter holes-on the surface. This allowed free movement of air from its two open holes on the top. The volunteers were requested not to touch or wash the dosed area for 24 h. (This was asked of all groups.)

97.2.3.2 Group B

A 50 cm^2 area (3.5 cm × 14.3 cm) was marked on the left and right ventral forearms of each volunteer. For each forearm the marked areas received a single topical application of total 0.17 g (22 μCi) [^{14}C]-estradiol gel formation using a 0.1 ml Teflon-coated syringe (Hamilton Company, Reno, NV). After dosing, the dosed area was allowed to air dry for 1 h after topical application. After 1 h, a Tyvek paper sleeve was placed on each dosed forearm for 24 h.

97.2.3.3 Group C

A 100 cm^2 area (5.0 cm × 20 cm) was marked on the left ventral forearm of group C volunteers. The marked area received a single topical application of 0.16 g (22 μCi) [^{14}C]-estradiol gel formulation using a 0.25 Teflon-coated syringe (Hamilton Company, Reno, NV).

After topical application, the dosed area was allowed to air dry for 1 h; then the ventral forearm (containing drug) was rubbed and brought in contact with the ventral forearm of group D volunteers for 15 min. After rubbing, the dosed forearm of group C volunteers was placed in a sleeve for 24 h. The volunteers were requested not to touch or wash the dosed area for 24 h.

97.2.3.4 Group D

A ventral forearm of group D volunteers was in contact with the ventral forearm (containing drug) of group C volunteers. Skin contact consisted of rubbing five times up and down in one direction (parallel to the axis of the arm), close contact of skin surfaces for 15 min, and rubbing five times up and down in the other direction. After rubbing, the contacted forearm of group D was placed in a sleeve for 24 h.

97.2.4 Skin Washing and Analysis

Twenty hours after dosing, the cover or sleeve was removed and each dosed site was washed using cotton balls (Sherwood Medical, St. Louis, MO), Ivory liquid soap (Procter & Gamble, Cincinnati, OH), and water. The water washing procedure was as follows:

1. 50% Ivory soap solution (v/v)
2. Distilled deionized water
3. 50% Ivory soap solution (v/v)
4. Distilled deionized water
5. Distilled deionized water

Each dosed skin area of groups A, B, and C volunteers was washed five times. The area outside of the dosed skin on the ventral forearm (called "nondosed skin site") of group C was also washed. Since the whole skin area on the ventral forearm of group D volunteers was considered to receive the topical formulation after rubbing, it was divided into two parts, the middle-upper and middle-lower of the ventral forearm. Each part was washed five times using the washing procedure described above. Individual cotton balls were placed in a borosilicate glass vial containing 5.0 ml of methanol overnight and then 10.0 ml scintillation cocktail was added. Appropriate dilutions were made on the basis of radioactivity. Radioactivity in these samples was analyzed by liquid scintillation counting (LSC) to quantify the amount of [^{14}C]-estradiol removed from the application site. The cover was also washed five times using the above procedure. The cotton balls were individually placed in a borosilicate glass vial with 10.0 ml of scintillation cocktail. Radioactivity of these samples was counted using the LSC.

97.2.5 Skin Tape Stripping and Analysis

The tape stripping on groups A and B was done 168 h after skin washing to analyze the residual dose. The dosed skin site was stripped 10 times with Scotch® cellophane tape 5912 clear (3M Commercial Supply Division, St. Paul, MN). These tapes were then individually placed in borosilicate glass vials with 5 ml of methanol overnight; then after addition of 10 ml of scintillation cocktail they were subsequently assayed for radioactivity by LSC. Tape stripping was not done on groups C and D because the skin rubbing sequence changed the original dosing area.

97.2.6 Radioactivity of Sleeve

The sleeve was cut into 20 pieces and individually placed in borosilicate glass vials with 5 ml of methanol overnight; then after addition of 10 ml of scintillation cocktail they were subsequently assayed for radioactivity by LSC.

97.2.7 Urine Sample Collection and Analysis

Urine samples were collected 0–24, 24–48, 48–72, 72–96, 96–120, 120–144, and 144–168 h after dosing. The volume of urine sample was measured and aliquots of urine sample (approximately 20 ml) were kept. Duplicate aliquots of 1.0 ml were used to determine radioactivity. Estradiol topical bioavailability was determined by urinary ^{14}C excretion and calculated relative to an estradiol human intravenous dose (Feldmann and Maibach, 1969) to determine absolute bioavailability.

97.2.8 Radioactivity Assay

All radioactivity measurements were conducted using a Model 1500 Liquid Scintillation Counter (Packard Instruments). The counter was audited for accuracy using sealed samples of quenched and unquenched standards as detailed by the instrument manual. Background control and test samples were counted in duplicate where possible for 3 or 5 min each. Weighed aliquots of urine, skin wash, tape strip, sleeve, and cover wash samples, skin, and receptor fluid were mixed directly with Universal Scintillation Cocktail (ICN Biomedicals, Costa Mesa, CA) and analyzed for radioactivity.

97.2.9 Statistical Analysis and Topical Bioavailability

Statistical analysis (*t*-test, one-way ANOVA) was done using SIGMASTAT (SPSS Inc., Chicago, IL). Topical bioavailability was calculated as urinary excretion (topical dose)/urinary excretion (i.v. dose) $\times$ 100. The data of urinary excretion following a single i.v. dose, 51.6% dose excretion, were cited from Feldmann and Maibach (1969).

97.3 RESULTS

Table 97.1 summarizes the clinical results. Urinary ^{14}C excretion was 3.9 $\pm$ 2.1, 4.2 $\pm$ 3.2, 3.4 $\pm$ 4.0, and 2.2 $\pm$ 2.0% applied dose for clinical groups A, B, C, and D, respectively. These urinary excretion calculated relative to an intravenous dose (Feldmann and Maibach, 1969) gave topical bioavailability as percent doses absorbed of 7.5 $\pm$ 4.1, 8.2 $\pm$ 6.3, 6.6 $\pm$ 7.6, and 4.3 $\pm$ 3.8 for groups A, B, C, and D, respectively. There were no statistical differences between these groups. The ^{14}C concentration in the urine samples decreased with half-lives of 28.4 $\pm$ 9.1, 30.8 $\pm$ 12.0, 29.3 $\pm$ 4.5, and 33.6 $\pm$ 13.1 h, respectively, for groups A, B, C, and D. There were no statistical differences between these small groups.

After the 24 h dosing period, the dosed skin site was washed with soap and water, and the skin was tape-stripped on day 7 for residual chemical. The protective cover or sleeve was also analyzed for residual chemical. Table 97.2 shows that 73–74% applied dose was recovered in the wash from group A after the 24 h dose period. There was no residual

TABLE 97.1
Clinical Group[a]

	Mean Percent Dose ± SD ($n = 6$)			
	A	B	C	D
Urinary ^{14}C excretion	3.9 ± 2.1	4.2 ± 3.2	3.4 ± 4.0	2.2 ± 2.0
Topical bioavailability[b,c]	7.5 ± 4.1	8.2 ± 6.3	6.6 ± 7.6	4.3 ± 3.8
T 1/2 Renal excretion (h)	28.4 ± 9.1	30.8 ± 12.0	29.3 ± 4.5	33.6 ± 13.1
Dose accountability[d]	82.0 ± 5.3	56.9 ± 11.0	56.9 ± 7.1	10.8 ± 7.9

[a] A = Dose site protected with nonocclusive cover ($n = 6$); B = Dose site not protected ($n = 6$); C = Skin site dosed, then rubbed on volunteers in group D ($n = 6$); D = Naive recipient of skin contact/rub ($n = 6$).

[b] Topical bioavailability = urinary excretion (topical dose)/urinary excretion (iv dose) × 100; 51.6% dose excreted after single i.v. human dose (Jewelewicz, 1997).

[c] No statistical difference ($p > .05$).

[d] Please note that mass balance is higher where topical dose was better controlled.

TABLE 97.2
Clinical Group—Treatment Forearm

		Mean Percent Dose + SD ($n = 6$)			
		A	B	C	D[a]
Wash	Left	73.0 ± 10.0	27.2 ± 4.0	36.1 ± 14.4	2.3 ± 2.0
24 h	Right	74.0 ± 6.1	29.0 ± 4.0		
Cover[b]	Left	0.9 ± 6.1[b]	24.2 ± 14.4[c]	12.9 ± 6.6[c]	4.1 ± 3.6[c]
Or					
Sleeve[c]	Right	1.2 ± 1.6[b]	13.7 ± 3.8[c]		
Tape Strip	Left	0.02 ± 0.01	0.05 ± 0.07		
Day 7	Right	0.02 ± 0.02	0.03 ± 0.03		

[a] The percent recovery in group D is based on the dose applied to group C.

[b] Cover is nonocclusive/non-skin-touching cover over the dosed site.

[c] Sleeve is a disposable cover placed over the arm to stop the spread of radioactivity to the volunteer's clothing and environment.

chemical in day 7 tape strips. Group B wash recovery was 27–29%, and 14–24% was transferred to the covering sleeve. There was no residual dose in the tape strips. Group C wash recovery was 36%, and 13% was recovered from the sleeve. Group D wash recovery was 2.3%, and 4.1% was recovered from the sleeve. Tape strips were not done on groups C and D because the dosing area could not be defined after the skin rub/contact. Dose accountability for the groups is listed in Table 97.1. The results show that the majority of the topical dose (groups A and B) resides on the surface of the skin during the 24 h dosing period. The dose is available for skin transfer (group C), and the skin transferred dose is absorbed by the recipient person (group D).

97.4 DISCUSSION

Topically applied chemicals reside on the skin for an extended dosing period, and these chemicals can transfer through skin contact with another naive person. This is probably of no consequence for inert chemicals; however, potent topical bioactive chemicals have become available. An example given previously was of topical testosterone on a father which could be transferred by contact to his son, as shown by this study. The testosterone cream was applied without attention to hand washing after application or the possibility of contact transmission to other family members. This began a few months after his son's birth (Franklin and Geffner, 2003). Different from testosterone that may induce inadvertent effects in immature and female individuals, no relevant risk scenario is currently known from possible transfer of 17 β-estradiol.

Our study, using single defined doses of labeled estradiol (about 0.1 mg) and demonstrating absorption rates <10%, was unlikely to produce systemic biological effects in this case. Topical drugs intended for systemic delivery are routinely used over an extended period of time. Our group A results show that 73−74% protected estradiol dose was still on the skin, and group B showed 27−29% dose still on the unprotected skin and 14−24% on the clothing (sleeve), probably all still available for absorption. Since the total bioavailability was 7.5% for group A and 8.2% for group B, a large portion of active substance is still available for transfer/absorption. This study does not answer the question of how long residual topical drug would be available on the skin for transfer relative to time, formulation changes, or effect of routine skin washing.

Topical bioavailability using cumulative urinary recovery of estradiol in this study was 7.5 ± 4.1 and 8.2 ± 6.3% doses for groups A and B, respectively. This compares favorably with the published *in vivo* estradiol percutaneous absorption of 10.6 ± 4.9% doses (Feldmann and Maibach, 1969). This study and the report by Franklin and Geffner (2003) document transfer of estradiol and testosterone. During discussions on doping in sports (BALCO, Olympics), the "cream" (testosterone) is mentioned as a contributing factor. There is a report cited earlier of suspected fentanyl transfer (Yerasi et al., 1997). This transfer potential will apply to any bioactive topical chemical, and this should not be restricted to drugs. Hazardous environmental chemicals (pesticides, herbicides, pollutants) can topically settle on one individual and be transferred to another person.

This study shows only the one rub/contact sequence for one time period after dosing for one formulation, but skin contact can occur under a variety of conditions, and each variable will contribute to the overall chemical skin transfer. More data are thus warranted. However, the potential for skin transfer of topical bioactive chemicals has been demonstrated *in vivo* in human volunteers and an awareness of this effect is warranted.

CONFLICT OF INTEREST STATEMENT

UCSF contracted with Dr. Kade (Dr. Kade Pharmazeutische Fabrik GMBH, Berlin, Germany) for this study. No other funds or conflict of interest occurred.

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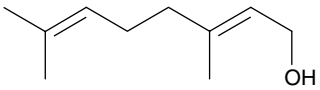
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98 Is There Evidence That Geraniol Causes Allergic Contact Dermatitis?

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98.1 INTRODUCTION

Geraniol is frequently used in cosmetics (ca. 70% of perfumes examined by Johansen and coworkers [1]) and has been cited as a moderately frequent cause of allergic contact dermatitis [2]. The following reviews the published data on the sensitization potential of geraniol: (*E*)-3,7-dimethyl-2,6-octadien-1-ol (CASRN: 106-24-1, EINECS 203-377-1). A semiquantitative evaluation of the different reports cited below has been made in accordance with the rules outlined by Hostynek and Maibach [3], which are based on those made by Benezra and others in 1985 [4]. This approach has already been used to examine four other substances that have also been cited as frequent causes of contact dermatitis: alpha-*iso*-methylionone [5], anisyl alcohol [6], linalool [7], and amlycinnamic aldehyde [8].

98.2 METHODS

The medical literature was searched using the electronic databases Biosis, Caplu, Embase, RTECS, Toxlit, Medline/HealthStar, Toxnet, and Science Citation Index (1960 to late 2004). Search terms included "geraniol," "allergic contact dermatitis," "sensitization," and "patch tests." Copies of all cited publications were obtained except for *Annali Italiani Dermatolgia Allergologia* and *Bolletino Dermatologia Allergologica e Professionale—Manuela Pangrazio* for which back copies were not available. The Research Institute for Fragrance Materials Inc. (RIFM) kindly made available copies of unpublished studies performed by its members or carried out under its commission. A hand search of nonentered literature (*Lettre de GERDA*, and textbooks of allergic dermatitis in English, German, Spanish, French, and Italian) was performed.

98.3 RESULTS

98.3.1 Data from Predictive Tests Carried out on Animals

Several animal tests have been reported. The degree of confidence that can be ascribed to these reports is given according to the criteria outlined by Hostynek and Maibach [3] (see Appendix A).

Several local lymph node assays (LLNAs) have been performed on geraniol. In one for which only a brief summary was reported, geraniol was found to be nonsensitizing [9]. A degree of confidence of two was given to this due to the lack of published detail. In another carried out at doses up to 50%, different indicators of allergenic activity were elevated

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compared to the vehicle control, but a stimulation index of 3 (criteria of positivity) was not reached [10]. A rating of 4 was given for this fully reported state-of-the-art study. Another LLNA was also negative [11]. A series of LLNAs were performed by RIFM to determine the effect of different solvents [12]. In one using ethanol as a solvent, the EC3 was calculated as about 6%. In another using diethyl phthalate as solvent, the EC3 was about 12%. When the solvent was a 3:1 mixture of diethyl phthalate and ethanol, the EC3 was just over 20%, but when these ratios were reversed (1:3 mixture of diethyl phthalate and ethanol), the EC3 was calculated as just over 25%. These studies were given a degree of confidence of 4 even though it is not certain that the differences between these EC3 values are significant. It can be concluded with a good degree of confidence that geraniol is a moderate-to-weak sensitizer according to these LLNA tests.

Guinea pig maximization tests have been carried out. Four tests using dermal induction levels of 50% gave no reactions in two of these [13]. In another, no reactions were seen after the first and third challenges but 2/10 guinea pigs reacted at the second challenge. In the fourth test, two reactions (out of nine) were seen after the first challenge, but only a questionable reaction was seen after the second challenge and no reaction was seen after the third challenge. Although the disappearing reactions are perplexing, these four tests inspired a high degree of confidence (4) even if the number of animals was low. In another guinea pig maximization test (closed epicutaneous test) performed at a lower concentration, a score of 0.5 was reported [14], but as this study was carried out under submaximized conditions it was given a rating of 2. A brief report of guinea pig maximization test on 10% geraniol indicated a score of 3/6 reactions [15]. This was given a rating of only 3 due to the extremely summarized manner in which this report was published. In the case of these guinea pig maximization tests, the tests that were most highly maximized and to which we attribute the highest degree of confidence showed that geraniol was capable of causing any significant reactions.

Other tests in guinea pigs gave mixed results. A Freund's complete adjuvant test gave one reaction in seven animals [15]. This test is reported only as a summary and hence a rating of 3 is accorded. An open epicutaneous test gave 3/8 reactions at 10%, 5/6 reactions at 30 and 100% [15]. Although the published report is highly summarized, a more detailed report was submitted to RIFM [16], allowing a degree of confidence of 4. Both of these tests were given a rating of 4. Two separate Draize sensitization screens in guinea pigs were completely negative [15,17] and being reported in summary form, only inspired a rating of 3. A Buehler test [18] in which geraniol was tested at 25% in diethyl phthalate was rated as 4 and was completely negative. These additional studies confirm that geraniol is a weak sensitizer in these assays.

98.3.2 Predictive Tests Performed on Human Volunteers

A human maximization test carried out on 25 volunteers at a concentration of 6% in petrolatum gave no reactions [19]. However, this study was probably carried out under submaximized conditions and is given a rating of 2. Human maximization tests carried out under the same conditions on Japanese-American subjects gave one possible reaction in 26 volunteer [20]. These studies were also rated at 2. A modified Draize sensitization screen of geraniol in ethanol at 10% was reported to give two reactions in 73 volunteers but none in 104 volunteers when tested at the same concentration in petrolatum [21]. The higher concentration allows a rating of 3 to be accorded to these. A human repeat insult patch test carried out at 5% in ethanol gave no reactions in 40 subjects [22]. Another human repeat insult patch test carried out at only 2% in ethanol/diethyl phthalate (1:3) also gave no reactions [23]. Both of these human repeat insult patch tests were rated at 2 due to the low dose. In summary, the seven human predictive studies indicate a weak or absent sensitization potential.

98.3.3 Clinical Elicitation Tests on Human Patients

A number of reviews have already been published on allergic reactions to geraniol [24–26]. One review states that the frequency of allergic reactions seen to geraniol has remained "relatively constant" over the 17-year period from 1980 to 1996 [27]. In another review of reactions seen in Japan over two periods, reactions to geraniol (rounded to the nearest percentage) in cosmetic-sensitive patients was 3% in 1971–1977 and 4% in 1978–1980, whereas in patients whose dermatitis was apparently unrelated to their use of cosmetics, these frequencies were 1 and 2%, respectively [28]. In another review of reactions in patients who had given positive reactions to the fragrance mix in Germany, the overall proportion for the period 1996–2002 was 5.9% [29]. The relative share of reactions to geraniol compared to the other components of the fragrance mix increased in 1999 but then subsequently decreased in the period 2000–2002. Concomitant reactions to geraniol and other fragrance mix components were frequent, particularly to hydroxycitronellal and oakmoss.

Owing to its presence as one of the eight components of the fragrance mix, there are, not unsurprisingly, numerous reports of studies in which geraniol has been used in routine patch testing. An attempt has been made to include all reported cases where reactions have been observed to geraniol. A more limited number of studies relate to an investigation of specific cases where patients had reacted to products containing this substance. All of these reports are examined here in the light of the criteria outlined by Hostynek and Maibach [3], which attempt to weigh the clinical relevance of the positive patch-test reactions (i.e., has geraniol caused the allergic dermatitis from which these patients were suffering?). The numerical grading system is shown in Appendix A.

98.3.3.1 Tests Producing Reactions

The following reports indicate positive elicitation reactions to geraniol.

One patient of 75 who reacted to fragrance and cosmetic ingredients (taken from 1781 patients over a 6-year period) also reacted to geraniol at an unspecified concentration

(probably 5%) [30]. Only 32 of these patients gave any reactions at all, but this group still gave 82 positive reactions. A rating of 2 was given to this publication, which is more than that given to a review of 11 publications by the same group.

Two patients of 119 who reacted to different fragrance and cosmetic ingredients also reacted to closed patches containing geraniol at 5% [31]. In fact only 53 patients reacted to any of the test materials but a total of 147 reactions were observed. This paper indicates that parts of these results have already been published by these authors in seven other reports, some of which are also reported in this Section 98.3.3.1 and in Table A2. These reactions, which were given a rating of 2, may therefore have been reported twice in this review.

One of two patients who had reacted to products containing citronella oil reacted to 1% geraniol [32]. In this study, however, stronger reactions were seen to citronellal at 1% and to some other substances. A rating of 2 was given as the evidence points to other components of citronella oil.

A patient who had reacted to citrus fruit peel and had given a positive reaction to citral also reacted to 5% geraniol [33]. A confidence level of only 2 was given because of the likelihood of a cross-reaction with the major peel component citral.

The causative role of geraniol was also implied in a case of a patient who had reacted to a cologne and who had subsequently reacted to the fragrance mix, rose oil, and 2% geraniol [34]. As cross-reactions with other components of rose oil (notably citronellol and citral) were not ruled out, a score of 3 was given.

The causative role of geraniol in an allergic reaction to a lip salve was decided on the basis of a positive reaction in the same patient to 2% geraniol [35]. A rating of 4 was given.

In a review of studies (also reported later ([59]) on 2461 eczema patients, 7 (0.2%) were found to have reacted to 2% geraniol [36]. No information was given on the severity of the reactions and there was no linkage to products containing geraniol that may have been responsible for these reactions. As a consequence, a degree of confidence of 2 was attributed.

Five out of 167 patients in a 3-year multicenter study exhibited what were classified as allergic reactions to 5% geraniol with seven patients showing irritant reactions [37]. This study showed a high degree of polyreactivity. The reacting patients reacted to an average of 4.5 of the test materials. A rating of 2 was given.

In a study on a mixed group of 50 healthy and eczematous patients (the latter being divided into those with cosmetic allergy and those with other allergies), a number of immediate reactions were seen in open testing to 5% geraniol but only one reaction was seen after closed patch testing [38]. This reaction of unknown severity was not associated with any particular product and was experienced by a patient in the group who had no history of cosmetic allergy. A rating of 2 was given.

In a study performed on 747 patients with "suspected fragrance allergies," seven reacted to 1% geraniol [39]. This study was associated with a high degree of polyreactivity. No information was given on the severity of the reactions and there was no linkage to products containing geraniol that may have been responsible for these reactions. As a consequence, a degree of confidence of 2 was attributed.

A study [40] showed that 6 out of 20 perfume-sensitive patients reacted to this substance when tested at 5%. However, this report indicated that these six patients all reacted to other test materials, most notably, hydroxycitronellal. No indications were given in relation to the relative severity of these different reactions. Later studies [37] have shown that 5% may be too high and produces irritant reactions. As a result, a degree of confidence of 2 was given.

In a study on a patient who had reacted to an ointment used to treat a leg ulcer, positive reactions were observed to the fragrance of the ointment and to 2% geraniol [41]. A rating of 3 was accorded because other possible causes had not been completely excluded.

Another study on two patients who also reacted to their ointments (one used to treat a leg ulcer and the other used to treat a burn), reactions were seen to an undisclosed concentration of geraniol [42]. A rating of 3 was accorded because other possible causes had not been completely excluded and the patients had reacted to other patch-test materials (fragrance mix and eugenol, respectively).

An occupational exposure to lemon peel gave allergic reactions in a bakery worker who failed to react to most components of lemon peel (citral, limonene, terpineol, linalool, citronellol) but who did react to 5% geraniol with an intensity that approximately commensurated with reactions to 10% lemon oil and 10% citronella oil [43]. The lack of test details and the absence of any test to citronellal reduce the degree of confidence to 3.

In a study on 5202 patients, 11 reacted to an undisclosed concentration of geraniol but only 2 of these reactions were experienced by patients with a history of cosmetic allergy [44]. Two hundred and fifty-seven reactions were experienced by 156 of these patients. No information was given on the severity of the reactions and there was no linkage to products containing geraniol that may have been responsible for these reactions. As a consequence, a degree of confidence of 2 was attributed.

In a 10-year study on reactions to the individual components of the fragrance mix in 283 fragrance mix–positive patients, 19 reactions were seen to 1% geraniol [45]. Two hundred and thirty-three reactions were experienced by the 133 patients who reacted to any of the components with 73 patients experiencing reactions to more than two components. No information was given on the severity of the reactions and there was no linkage to products containing geraniol that may have been responsible for these reactions. As a consequence, a degree of confidence of 2 was attributed.

In another study of reactions to the individual components of the fragrance mix in 50 fragrance mix–positive patients, 2 delayed-type reactions were seen to an unknown concentration of geraniol [46]. Thirty-one reactions were experienced by the 20 patients who reacted to any of the components. No information was given on the severity of the reactions and there was no linkage to products containing geraniol that may have been responsible for these reactions. As a consequence, a degree of confidence of 2 was attributed.

In a 5-year multicenter study involving tests on 713 patients with cosmetics dermatitis, 87 individual ingredients were patch tested on 53% of these (378 patients), giving eight cutaneous reactions to geraniol at an unspecified concentration (81% of all observed reactions were judged to be genuine sensitization reactions) [47]. No information was given on the severity of the reactions and there was no linkage to products containing geraniol that may have been responsible for these reactions. As a consequence, a degree of confidence of 2 was attributed.

Another multicenter study in Indonesia showed that 2 of a group of 17 fragrance mix–positive patients reacted to 1% geraniol [48]. No information was given on the severity of the reactions and there was no linkage to products containing geraniol that may have been responsible for these reactions. As a consequence, a degree of confidence of 2 was attributed.

Patch testing over 15 years on 41 patients gave one reaction to 1% geraniol [49]. The same study examined 16 other possible allergens and the total number of reactions exceeded the number of patients indicating that some reacted to more than one substance. No information was given on the severity of the reactions and there was no linkage to products containing geraniol that may have been responsible for these reactions. As a consequence, a degree of confidence of 2 was attributed.

In a study on 20 patients who were sensitive to the fragrance mix, 2 gave delayed-type reactions when patch-tested with 2% geraniol, whereas another eight suffered immediate-type urticarial reactions [50]. The two patients experienced reactions to three and five other components of the fragrance mix (in some cases with more severe reactions). There was no linkage to products containing geraniol that may have been responsible for causing these allergies. As a consequence, a degree of confidence of 2 was attributed.

In a study on 5315 consecutive patients, 299 were found to be sensitive to the fragrance mix and of these, 42 were further tested with 35 essential oils and with the 8 components of the fragrance mix. Ten patients reacted to 2% geraniol [51]. One hundred and seventy-four reactions were experienced by the 42 patients to the 35 essential oils and 89 reactions were experienced to the eight components of the fragrance mix. These 10 patients may have reacted to other substances and essential oils. No information was given on the severity of the reactions and there was no linkage to products containing geraniol that may have been responsible for these reactions. As a consequence, a degree of confidence of 2 was attributed.

Three out of seven fragrance mix–sensitive patients reacted to 2% geraniol [52]. These patients reacted, however, to 17, 22, and 14 different substances and essential oils as well. No information was given on the severity of the reactions and there was no linkage to products containing geraniol that may have been responsible for these reactions. As a consequence, a degree of confidence of 2 was attributed.

In a multicenter study on the effects of sorbitan sesquioleate (SSO), 702 patients were tested with 1% geraniol with and without SSO [53]. In the absence of SSO, three patients gave allergic reactions to geraniol, whereas five experienced allergic reactions to geraniol mixed with SSO. Six patients who had experienced doubtful or irritant reactions to geraniol and SSO failed to react to geraniol in the absence of SSO. Repeated open application testing (ROAT) of two of the patients who had reacted to geraniol failed to confirm positivity to geraniol. A confidence rating of 2 was ascribed to this report as an indication of the causality of geraniol.

Although a multicenter study failed to show reactions to geraniol at 1 and 0.1%, this report [54] cites the results of testing 1072 patients in nine participating clinics with the components of the fragrance mix. These results, which may include positives reported elsewhere, indicate that eight patients gave positive reactions to 1% geraniol in the presence of SSO. A further four patients gave doubtful/irritant reactions. The data are by necessity summarized and not linked to possibly causative products. A confidence rating of 2 was ascribed to this report as an indication of the causality of geraniol.

When 162 patients who were sensitive to the fragrance mix were tested to their individual components, 69 of them gave reactions [55]. Only four of these reacted to 1% geraniol and these patients also experienced reactions to the fragrance mix and a further 10 reactions to the other components indicating a high degree of polyreactivity. The data are highly summarized and not linked to possibly causative products. A confidence rating of 2 was ascribed to this report as an indication of the causality of geraniol.

In a study on the causes of eyelid dermatitis, 19 patients suffering from this condition were patch tested to 25 fragrance materials and gave no positive reactions to 2% geraniol [56]. Tests on 51 cases of dermatitis at other sites gave two reactions to the same material. The data are highly summarized with no indication of severity of the reactions being given and these are not linked to possibly causative products. A confidence rating of 2 was ascribed to this report as an indication of the causality of geraniol.

Patch testing on 40 patients to 2% geraniol gave one reaction [57]. The same study examined 27 other possible allergens and the total number of reactions exceeded the number of patients indicating that some reacted to more than one substance. No information was given on the severity of the reactions and there was no linkage to products containing geraniol that may have been responsible for these reactions. As a consequence, a degree of confidence of 2 was attributed.

In a summary of patch testing at St. John's Hospital in which patch testing was performed on 2461 patients in 1979–1980 and 1836 patients in 1984 (results which have also have been reported by other workers such as Lavsen and Maibach [36] and Calnan and coworkers [58]), 7 patients reacted to 2% geraniol in the first period and 10 reacted in the second [59]. No information was given on the severity of the reactions and there was no linkage to products containing geraniol that may have been responsible for these reactions. As a consequence, a degree of confidence of 2 was attributed.

A study of 15 patients sensitive to Peru balsam showed two reactions to 10% geraniol and one reaction in 253 normal

controls [60]. No information was given on the severity of the reactions particularly with regard to the patient's reaction to Peru balsam. There was no linkage to products containing geraniol that may have been responsible for these reactions. As a consequence, a degree of confidence of 2 was attributed.

Patch-test and photopatch-test studies in Japan over 1 year showed that 5% geraniol produced one reaction in 111 patients in normal patch testing and no reactions in photopatch testing on the same patients [61]. Although the frequency of reactions to different types of products is given, there is no linkage to the products containing geraniol that may have been responsible for these reactions. As a consequence, a degree of confidence of 2 was attributed.

In another study using 5% geraniol, 3 of 64 cosmetic-sensitive patients reacted, whereas 1 out of 32 patients with dermatitis unrelated to cosmetics reacted and no normal subjects in a control group reacted [62]. No information was given on the severity of the reactions and there was no linkage to products containing geraniol that may have been responsible for these reactions. As a consequence, a degree of confidence of 2 was attributed.

In a 6-year study in Japan on patients who had suffered from pigmented allergic dermatitis, 7 patients out of 123 (presumably not the same patients returning each year) reacted to the surprisingly high level of 20% geraniol [63]. Here too, there is insufficient evidence to establish a clear causal link between these reactions and the condition of the patients. A rating of 2 was attributed.

In further studies in Japan [64], geraniol at 5 and 2% failed to elicit reactions in 45 patients suffering from melanosis facea. However, in a group of 120 patients suffering from cosmetics-related dermatitis, 2 reacted to 5% geraniol but none reacted to 2%. Similarly, in a group of 78 patients suffering from cosmetics-unrelated dermatitis, one reacted to 5% geraniol but none reacted to 2% geraniol [64]. The disappearance of reactions at the lower concentration erodes the degree of confidence further in addition to the absence of information on potential causative products (rating of 2).

Of 23 patients who gave positive reactions when patch tested with a fragrance formulation containing geraniol, only 3 reacted to this substance when tested at 1% [65]. Cross-reactions were also seen with ylang ylang oil and benzyl salicylate. As a consequence, a degree of confidence of 2 was attributed.

A patient who reacted to a roll on deodorant, reacted also to the fragrance and a fraction of the fragrance containing lilial and geraniol; subsequently, reacting to both of these substances on their own at 1% [66]. Reactions to lilial were more intense and of longer duration than to geraniol. It was concluded that the causative agent was lilial because the allergy to geraniol was apparently developed during the patch testing. The reaction to geraniol was concluded as being not clinically relevant and a rating of 1 was therefore given.

One patient of 242 reacted to geraniol at 1% [67]. This reaction was not linked to any potentially causative product and a rating of 2 was ascribed.

In another study on facial melanosis in Japan, 35 patients with this condition failed to react to 5% geraniol [68]. However, 2 patients from a group of 212 with cosmetics-related dermatitis and 1 from a group of 275 with cosmetics-unrelated dermatitis did react. A control group of 101 normal subjects failed to produce any reactions to this substance. There was no linkage to products containing geraniol that may have been responsible for these reactions. As a consequence, a degree of confidence of 2 was attributed.

Studies on 1200 dermatitis patients in Italy in 1983–1984 showed that of 63 patients who reacted to the fragrance mix, 43 reacted to at least one of the individual components (giving 77 reactions) and of these, 4 reacted to geraniol at 3% [69]. In the second series involving 1500 patients in 1984–1985, 54 reacted to the fragrance mix and 45 of these reacted to at least one of the individual components (58 reactions) and of these, 1 reaction was to geraniol at 1%. In view of the polyreactivity, the causality of geraniol in these cases could not be clearly established. Furthermore, there was no linkage to causative products containing geraniol, allowing a rating of 2 to be attributed.

In studies on 757 patients of whom 112 reacted to the fragrance mix, 50 of these reacted to the individual components of this mix and of these, 3 reacted to 2% geraniol in petrolatum with SSO [70]. There was some degree of polyreactivity because these 50 patients experienced 75 reactions to these components. Again, there was no linkage to causative products that may have contained geraniol and hence a rating of 2 was given to this report.

In a study on 4975 consecutive patients, 407 reacted to the fragrance mix and from these, 38 patients were tested with the individual ingredients giving 5 reactions to 1% geraniol [71]. In fact only 32 of these subjects reacted to any of the components, giving rise to 75 reactions. In view of this degree of polyreactivity and as no linkage to causative products that may have contained geraniol was made, a rating of 2 was given to this report.

In a multicenter study in Hungary, 12 of 160 fragrance mix–sensitive patients reacted to an unspecified concentration of geraniol [72]. A confidence level of 2 was ascribed because no evidence has been provided of the possible causative role of geraniol.

In a study on 241 patients, 10 reacted to 2% geraniol (out of 147 reactions to different fragrance materials experienced by this group [73]). This study was not designed to determine the causative allergens and was considered to attribute causality for these patients' allergies with a degree of confidence of 2.

In a prospective study of cosmetic reactions from 1977 to 1980, 487 patients gave 5 reactions (out of 338 reactions observed) to an unspecified concentration (probably 2%) of geraniol [74]. These positive reactions may also be taken up in the report of Adams and Maibach [47], and like their report (although it summarizes some detailed investigations that linked allergies to specific products through provocative product use tests), this one was also attributed with a rating of 2.

In a study on 2461 consecutive patients during 15 months, 7 reactions were ascribed to 2% geraniol [58]. Only 172 patients reacted to the fragrance mix but 338 reactions were seen to its eight constituents. Although the authors state that "most of the positive reactions were relevant to the patient's present or past dermatitis," insufficient information is given in this report to substantiate this and as a result, a rating of 2 was ascribed.

In a study of patch-test reactions over 14 years (1979–1992), 8215 consecutive patients were tested and gave 449 positive reactions to the fragrance mix. When 367 of these patients were tested with the individual constituents of the fragrance mix, 15 reacted to geraniol at 2% until 1984 and from then on at 1%, with a further 14 doubtful reactions [75]. A total of 338 positive and 142 doubtful reactions were observed in the 224 patients who reacted to one or more of these constituents. No linkage is made to possibly causative products and this report only provides a level of confidence of 2 in determining that geraniol was the causative agent.

Another study on 200 fragrance mix–sensitive subjects drawn from 1967 dermatitis patients produced 14 reactions (out of 310 observed when seven of the eight constituents of the fragrance mix were tested individually) to geraniol at a concentration of 1 or 3% [76]. In view of the polyreactivity and the absence of any established linkage of these reactions to causative effects or products, a low level of confidence (2) can only be ascribed.

In 61 patients out of 677 patch tested, 61 reacted to the fragrance mix and when these were tested with the eight individual constituents, eight reacted to 5% geraniol (out of 111 reactions recorded for the different constituents) [77]. Although this study revealed some interesting aspects of patch testing, it can only be given a level of confidence of 2 in attributing direct causality to geraniol for the allergies detected in these eight patients.

In a study on dentists and dental nurses in which 180 patch-test reactions were observed in 72 female dentists who had presented cases of suspected occupational allergic contact dermatitis, one reaction was seen to geraniol. No reactions were seen in dental nurses [78]. It is not known whether this patient reacted to other materials or whether any attempt was made to assess the clinical relevance of this case. For this reason, a rating of 2 was ascribed.

In a study on 179 cosmetic-sensitive patients, 67 reacted to 22 substances (giving 116 reactions), including 11 to a high concentration of geraniol (10%) [79]. There was clearly a high degree of polyreactivity and the authors discount the high concentration as a cause for the relatively high frequency of reactions as 11/179 is too low to represent false positives. No linkage was made to possibly causative products and this report only provides a level of confidence of 2 in determining that geraniol was the causative agent.

In a summary of 4 years of patch testing in 23,660 patients, of whom 1811 reacted to the fragrance mix, patch testing of the individual components of this mix on 1112 of these patients gave rise to 1294 reactions in 934 patients who reacted to at least one of these constituents. Patches containing 1% geraniol and 1% SSO produced 67 reactions to geraniol (SSO on its own at 1% in petrolatum produced 35 reactions) [80]. A degree of confidence of 2 was ascribed to this study that did not detail any results which linked these reactions to causative products.

Examination of the results of patch testing 3037 out of 8230 consecutive patients with the fragrance mix between 1968 and 1983 led to further tests on 144 fragrance mix–positive patients using the 8 individual components. Of these 10 reactions (out of 133 to the 123 patients who reacted to one or more constituents) to 1% geraniol [81]. No record was made of linkage of any of these reactions to causative products. As a result, a rating of 2 has been ascribed.

In a study aimed more at investigating possible causes of phototsensitivity dermatitis with actinic reticuloid syndrome, 3 subjects out of 457 reacted to 2% geraniol [82]. In all, 47 patients experienced a total of 72 reactions. No link was made to possible causes and as a result, a level of confidence of 2 has been ascribed.

A total of 5 reactions to geraniol in 31 oakmoss-sensitive patients were ascribed by the authors as concomitant positive reactions [83]. Furthermore, these 31 patients experienced 75 reactions to other substances. This was also ascribed a rating of 2.

Two patients who had suffered reactions to specific qualities of essential oils used in aromatherapy were found to react to 2% geraniol [84]. One of these patients reacted to 6 other substances (reacting more strongly to 3 of these than to geraniol) and to 32 essential oils (reacting more strongly to 19 of these). Twenty-one of these essential oils did not contain detectable levels of geraniol. The other patient reacted to 5 other substances (more strongly to 1 than to geraniol) and 21 essential oils (more strongly to 7). Most of her essential oils were not analyzed for the presence of geraniol but nine oils that had produced reactions contained no detectable levels of geraniol. In view of the number of reactions each patient produced to other substances or to oils containing no detectable geraniol levels, only a rating of 2 was ascribed.

In a review of testing in 31 clinics in Germany on 4900 patients over 4 years [85], 20 patients out of 566 who had given a 1+ reaction to the fragrance mix reacted to 1% geraniol. A further 34 out of another 425 patients who had given 2+ or 3+ reactions to the fragrance mix reacted to the same substance, but only 8 of these gave more than a 1+ reaction to geraniol. It is not clear from these studies whether geraniol had any responsibility at all for the cause of the patients' allergies, and for this reason a score of 2 has been attributed.

In a study on 133 fragrance mix–sensitive patients drawn from 2600 patients who had been routinely patch tested over a 10-year period, 19 reactions (out of 233 reactions observed when the individual components of the fragrance mix were tested) were to geraniol at 1% [86]. No linkage was made to specific products containing this ingredient and 233 reactions were observed in only 133 patients, leading to a level of 2 being ascribed. These same results were reported in another paper cited here [45].

In a study on 542 fragrance mix–sensitive patients drawn from 12,118 patients who had been routinely patch tested in

10 centers, 58 reactions (out of 623 reactions observed when the individual components of the fragrance mix were tested) were to geraniol at an unknown concentration [87]. No linkage was made to specific products containing this ingredient and the possibility of cross-reactions was not ruled out. Furthermore, 623 reactions were observed in only 542 patients, leading to a level of 2 being ascribed.

A small proportion (0.4%) of a group of 792 eczematous patients (presumably 3) reacted to 10% geraniol [88]. The lack of details about possible concurrent reactions suffered by these patients as well as any linkage to past exposure to particular products allows a rating of only 2.

The role of geranium oil in causing an allergic reaction to a lip balm containing this oil has been strongly indicated by a positive reaction to 5% geranium oil [89]. A rating of 3 was given because the role of geraniol (only one of many constituents of geranium oil) was not confirmed.

The ability of geraniol to cause urticarial reactions has only been indicated in one case where a patient reacted to an unspecified concentration of geraniol in an open test [90].

98.3.3.2 Tests Producing No Reactions

A number of studies on patients failed to produce positive reactions to geraniol when this was one of the patch-tested substances. A nonexhaustive list is provided here.

Tests carried out at a concentration of 1%: unspecified number of patients [91], 0/667 [92], 0/8 [93], 0/47 [94], and 0/170 [95]. Tests carried out at 2%: 0/336 [96]. Tests carried out at 5%: 0/115 [97] and 0/122 [98]. A score of 0/242 was obtained when testing patients to an unspecified concentration of geraniol [100].

Subjects who had been presensitized in predictive human repeated insult patch testing and who subsequently used shampoos containing geraniol, failed to react after 2 weeks exposure at levels of this substance that represented at first 30% of their "elicitation threshold" (from 48-h closed patch tests) and then 90% of this level [101].

98.4 DISCUSSION

Qualifying substances on the basis of graded evidence is widely used in preclinical testing and in the classification and designation of chemicals as skin sensitizers [102,103]. Rarely heeded attempts have been made to grade possible clinical relevance [104–106] in patch testing on patients. In this review, there is evidence from predictive studies that geraniol is a weak contact allergen, and there is an abundance of indirect clinical evidence (scores of 1–3) showing that geraniol is indeed an allergen in patients. Interestingly, there are no studies (with scores of 4 or 5) that clearly show that geraniol has caused a reported allergy that has brought a patient to the clinic. Indeed, no single study has investigated cases where patients have reacted to specific products containing geraniol and where the subsequent positive patch test has clearly attributed the allergy to geraniol. Nearly all of the clinical studies reported here have been aimed at goals other than simply determining the original cause of the patients' allergies and have often involved well-designed studies aimed at determining the relationship between positive reactions to the fragrance mix and reactions to its individual constituents. Others are simply clinical reports in which patients were subjected to multiple patch testing and are aimed more at determining the frequency of reaction to the substance and most have not attempted to establish clinical relevance according to currently accepted procedures [104–106]. This is not a criticism of most of these papers that elegantly achieved other goals. They did not set out to determine the role played by geraniol in the etiology of the patient's disease and hence we are unable to attribute any real degree of confidence to these papers as pointing to the responsibility of geraniol in this regard. It is possible that allergy to geraniol is quite widespread as indicated by the number of reported positive patch-test reactions. However, the frequency of these reactions is not an accurate indication of the frequency of incidents in which geraniol has been established as the direct cause of contact dermatitis resulting from the use of consumer products and other everyday environmental contact. A recent publication [107] relative to the testing of methyldibromo glutaronitrile–sensitive patients has discussed another aspect of patch testing. The use of patch-test concentrations that are too high (as the current levels used for geraniol may well be) decreases specificity without greatly affecting sensitivity. A recently published review of the dose–response relationships of elicitation as determined by the conditions of induction may offer an explanation for this phenomenon [108]. Consumers exposed to low doses in everyday life may acquire allergies that are effectively benign in that they have elicitation thresholds that are not transgressed anywhere but during patch testing.

98.5 CONCLUSIONS

Data from predictive tests in animals do not appear to indicate that geraniol is a particularly potent skin sensitizer. Predictive tests in humans were generally negative, although some reactions were obtained when ethanol was used in closed chambers. The possible potentiating effect of occluded ethanol (a situation that is not encountered during normal conditions of use of cosmetics) needs further elucidation. A number of clinical reports describe reactions to different concentrations of geraniol in multiple-substance patch testing on prior-sensitized patients. However, these studies are by their nature incapable of unambiguously designating geraniol as the cause of the patient's condition. Although its inclusion in cosmetic labeling declarations [109] and routine patch-testing materials as the fragrance mix may provide further information on its possible causal role in allergic contact dermatitis, the evidence currently available does not indicate that geraniol is a major fragrance allergen. Indeed it would seem that it has only a very weak allergenic potential and may be only rarely responsible for the allergies suffered by patients who subsequently react positively to this substance in patch testing.

Determining the clinical relevance of fragrance patch-test positivity presents a challenge to physicians and dermatologists. This should be simplified when more data become available about appropriate nonirritant patch-test concentrations and vehicles together with clinical correlations to Provocative Use Test/Repeat Open Application Tests (PUT/ROAT) [110,111]. Recent expedited schema for providing fragrance allergens should be of value [112].

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APPENDIX A

Assignment of Degree of Confidence

Test Qualification	Degree of Confidence
Meets all criteria	5
Number of cases is marginal	4
Some parameters questionable	3
Lack of controls; no corroborating results	2
Unreliable results	1
Fails all criteria	0

Note: Criteria: vehicle-treated and untreated controls, test concentration sufficient for response, use of appropriate vehicle, adequate compound purity, and significant number of cases used.

TABLE A1
Predictive Testing of Geraniol

Reference	Test Material Identified	Type of Test	Test Conditions Provided?	Was Test Fully Maximized?	Were There Adequate Controls?	Was Number of Subjects Sufficient?	Were Results Presented in Adequate Detail?	Final Rating
[8]	No data	LLNA	No data	No data	No data	No data	No	2
[9]	Yes	LLNA	Yes	Yes	Yes	OECD	Yes	4
[10]	No data	LLNA	No data	No data	No data	OECD	No	2
[11]	Yes	LLNA	Yes	Yes	Yes	OECD	Yes	4
[12]	No data	GPMT	Yes	Yes	Yes	OECD	Yes	4
[13]	No data	CET	No data	No data	No data	No data	No	2
[14]	No data	GPMT	Yes	Yes	No data	Only 6	Yes	3
[14]	No data	OET	Yes	Yes	No data	8/dose	Yes	3
[14]	No data	Draize	Yes	Yes	No data	8	Yes	3
[15]	No data	OET	Yes	At 6% only	No data	8/dose	No	3
[16]	"Commercial"	Draize	Yes	Yes	Yes	10	Yes	3
[17]	Yes	Buehler	Yes	Yes	Yes	20	Yes	3
[18]	No data	HMT	Yes	No, at 6%	No data	25	No	2
[19]	No data	HMT	Yes	No, at 6%	No data	24 and 26	Yes	2
[20]	Semipure	HRIPT	Yes	No, at 10%	No data	104 and 73	No	3
[21]	No data	HRIPT	Yes	No, at 5%	No data	40	Yes	2
[22]	No data	HRIPT	Yes	No, at 2%	No data	110	Yes	2

Note: LLNA = local lymph node assay; GPMT = guinea pig maximization test; OET = open epicutaneous test; CET = closed epicutaneous test; Draize = Draize guinea pig sensitization test; Buehler = Buehler guinea pig test; HMT = human maximization test; HRIPT = modified Draize/human repeat insult patch test; OECD = according to OECD guidelines.

TABLE A2
Diagnostic Patch Tests Implicating Geraniol

Reference	Primary Report	Number and Condition of Patients	Conditions of Patch Testing Given	Patients Reacting Scores	Has Irritancy Been Excluded?	Reaction to Other Materials?	Cross-Reactions Likely?	Excited Skin Excluded?	ROAT or PUT Used	Linkage to Specific Product	Final Score
[30]	Review	75: Ecz	No Dose, V = ?	1: score ?	No score ?	82 in 30	Poss	No	Cosmetic only	Coinc.	2
[31]	Multi. Cent.	119: CosmA	No 5%: Pet	2: score ?	No score ?	147 in 53	Poss	No	Cosmetic only	No	2
[32]	Primary	3: Ecz	Yes 1%: Acet	1: + to ++	Poss	11 in 2	Citronellal	No	No	Yes Citronella	2
[33]	Primary	1: Ecz	Yes 5%: Mineral oil	1: ++	No score ?	Yes	citral	No	No	Citrus peel	2
[34]	Primary	1: Ecz	No 2%: Pet	1: score ?	No score ?	Rose oil and FM	Poss	No	No	Cologne	3
[35]	Primary	1: Ecz	No 2%:Pet	1: +	No score ?	No	Poss	No	No	Lip-salve	4
[59]	Review	2461: Ecz	No 2%: Pet	7/2461, 10/1836: score ?	No score ?	Not tested	Poss	No	No	No	2
[37]	Primary	167: Frag A	Yes 5%: Pet	5: score ? 7: irr	No score ?	404 in 89	Poss	No	No	No	2

continued

TABLE A2 (continued)

Reference	Primary Report	Number and Condition of Patients	Conditions of Patch Testing Given	Patients Reacting Scores	Has Irritancy Been Excluded?	Reaction to Other Materials?	Cross-Reactions Likely?	Excited Skin Excluded?	ROAT or PUT Used	Linkage to Specific Product	Final Score
[38]	Primary	50: CosmA	Yes 5%: Pet	20: urticaria 1: score ?	No	293 in 50	Poss	No	**No**	No	2
[39]	Primary	747: FM+	No 1%: Pet/SS0	7: score ?	No score ?	22/patient	Poss	No	**No**	No	2
[40]	Primary	20: Ecz	Yes 5%: Pet	6: score ?	No score ?	3–6/patient	Poss	Phased Tasting	**No**	No	2
[41]	Primary	1: leg ulcer	No 2%: Pet	1: + +	No score ?	Not tested	Poss	No	**No**	Ointments	3
[42]	Primary	1: leg ulcer 1: burn	No Con/V =?	1: + + + 1: score ?	No score ?	FM (+ + +) Eugenol	Poss	No	No	Ointments Ointments	3 3
[43]	Primary	1: Ecz	No 5%: DEP	1: + + +	Poss	Citronella oil (contains Geraniol)	No	No	No	No	3
[44]	Primary	5202:Ecz 156: CosmA	No Conc/V = ?	11: score ?	No score ?	257: in 156	Poss	No	No	No	2
[45]	Primary	283: FM +	No 1%: Pet	19: score ?	No score ?	233 in 133	Poss	No	No	No	2
[46]	Primary	50: FM +	No Conc/V = ?	2: Delayed 1: Immediate	Poss for 2	31 in 20	Poss	No	No	No	2
[47]	Multi. Cent.	713: CosmA	Yes Conc/V = ?	8: cutaneous reactions score ?	No 19% nonallergic score ?	536 in 378	Poss	No	PUTs link with products No details	PUTs link with products No details	2
[48]	Multicenter	17: FM+	Yes 1%: Pet	2/17: score ?	No score ?	Not tested	Poss	No	No	No	2
[49]	Primary	41: Ecz	Yes 1%: Pet	1: score ?	No score ?	122 in 59	Poss	No	No	No	2
[50]	Primary	20: Frag A	Yes 2%: Pet	2: + +	Poss	3 for one patient, 5 for the other	Stronger reactions to other materials	No	No	No	2
[51]	Primary	5315: Ecz 299: FM +	No 2%: Pet	10: score ?	No score ?	263 in 42	Poss	No	No	No	2
[52]	Primary	7: FM +	No 20%: Pet	3: score ?	No score ? Concentration!	19 for one, 24 for another and 16 for third	Farnesol and others Poss	No	No	No	2
[53]	Multi. Cent.	702: Ecz	Yes 1%: Pet	5 with SS0, 3 without SS0 score ?	No score ?	Poss	Poss	No	ROAT negative to Geraniol	No	2
[54]	Multi. Cent.	1072: Ecz	Yes 1%: Pet	4: irritant or 1/8: + to + + +	Poss for reactions > + +	Poss	Poss	No	No	No	2
[55]	Primary	162: FM +	No 1%: Pet	4: score ?	No score ?	Poss	Poss	No	No	No	2

TABLE A2 (continued)

Reference	Primary Report	Number and Condition of Patients	Conditions of Patch Testing Given	Patients Reacting Scores	Has Irritancy Been Excluded?	Reaction to Other Materials?	Cross-Reactions Likely?	Excited Skin Excluded?	ROAT or PUT Used	Linkage to Specific Product	Final Score
[56]	Primary	51: Ecz	Yes 2%: Pet	2: score ?	No score ?	50–12 substances	Poss	No	No	No	2
[57]	Primary	40: Frag A	Yes 2%: Pet	1: score ?	No score ?	338–28 substances	Poss	No	No	No	2
[58]	Primary	2461: Ecz 172: FM +	No 2%: Pet	7: score ?	No score ?	305 in 172	Poss	No	No	No	2
[60]	Primary	15:Ecz Peru Balsam +	Yes 10%: Pet	2: score ?	No score ?	Poss	Poss	No	No	No	2
[61]	Primary	111: Ecz	Yes 5%: Pet	1: score ?	No score ?	98 to 17	Poss	No	No	No	2
[62]	Primary	64: CosmA 32: Ecz	No 5%: Pet	3/64: score ? 1/32: score ?	No score ?	Poss	Poss	No	No	No	2
[63]	Primary	123: Ecz	No 20%: Pet	7: score ?	No score ?	Poss	Poss	No	No	No	2
[64]	Primary	120: CosmA 78: Ecz	No 2%, 5%: Pet	2/120 at 5%, 1/78 at 5% score ?	No 0/120 at 2%, 0/78 at 2%	Poss	Poss	No	No	No	2
[65]	Primary	23: frag sens	Yes 1%: Pet	3: score ?	No score ?	Ylang ylang and benzyl salicylate	Poss	No	No	No	2
[66]	Primary	1: Frag A	No 1%: Pet	1: + at 48 h	Poss score = ++ at 24 h	Lilial +++	No	Proposed	No	No	1
[67]	Primary	242: Ecz	No 1%: Pet	1: score ?	No score ?	Poss	No	No	No	No	2
[68]	Primary	212: CosmA 275: Ecz	Yes 5%: Pet	2/212, 1/275 score ?	No score ?	Poss	Poss	No	No	No	2
[69]	Primary	1200 and 1500 FM +	3% (1200), 1% (1500): Pet	4/1200, 4/500 score ?	No score ?	77 in 48 and 58 in 45	Poss	No	No	No	2
[70]	Primary	757: Ecz 112: FM+	No 2%: Pet	3: score ?	No score ?	75 in 50	Poss	No	No	No	2
[71]	Primary	38 FM +	Yes 1%: Pet	5: score ?	Delayed reactions	75 in 32	Poss	No	No	No	2
[72]	Multicenter	160: FM+	No Con/V = ?	12: score ?	No score ?	Not tested	Poss	No	No	No	2
[73]	Primary	241:Ecz	Yes 2%: Pet	10: ++ or more	Poss score = ++	Poss	Poss	No	No	No	2
[74]	Multi. Cent.	487: CosmA	Yes Conc/V = ?	5: score ?	No score ?	Poss	Poss	No	PUTs on cosmetics	No	2

continued

TABLE A2 (continued)

Reference	Primary Report	Number and Condition of Patients	Conditions of Patch Testing Given	Patients Reacting Scores	Has Irritancy Been Excluded?	Reaction to Other Materials?	Cross-Reactions Likely?	Excited Skin Excluded?	ROAT or PUT Used	Linkage to Specific Product	Final Score
[75]	Primary	449: FM +	Yes 2%, 1%: Pet	15: positive 14: doubtful 0: irritant	Poss	338 in 224	Positive correlation with hydroxy-citronellal	No	No	No	2
[76]	Primary	200: FM +	Yes 1–3%: Pet	14: score ?	No score ?	310 in 200	Poss	No	No	No	2
[77]	Primary	677: Ecz 61: FM +	No 5%: Pet	8: score ?	No score ?	111 in 61	Poss	No	No	No	2
[78]	Primary	72: Ecz	Yes Con/V = ?	1: score ?	No score ?	180 in 72	Poss	No	No	No	2
[79]	Primary	179: Ecz	No 10%: Pet	11: score ?	No Concentration score ?	116 in 67	Poss	No	No	No	2
[80]	Primary	1112: FM +	No 1%: Pet	67: score ?	No score? 35 + to SS0	1294 in 934	Poss	No	No	No	2
[81]	Primary	144: FM +	No 1%: Pet	10: score ?	No score ?	133 in 123	Poss	No	No	No	2
[82]	Primary	457: Ecz	Yes 2%: Pet	3: score ?	No score ?	72 in 47	Poss	No	No	No	2
[83]	Primary	31: oakmoss +	Yes Con/V = ?	5: score ?	Score ?	80 in 31	Poss	No	No	No	2
[84]	Primary	2: aroma therapy +	No 2%: Pet	2: one +, one + +	Poss	38 in one 26 in other	Stronger reactions from other substances	No	No	No	2
[85]	Multi. Cent.	991: FM +	Yes 1%: Pet	52: + 8: + +	+ may be irritant	917 in 533	Poss	No	No	No	2
[86]	10-year review	133: FM +	Yes 1%: Pet	19: score ?	No score ?	233 in 133	Poss	No	No	No	2
[87]	10-center study	542: FM +	Yes ?: Pet	58: score ?	No score ?	623 in 542	Poss	No	No	No	2
[88]	Primary	792: Ecz	Yes 10%: Pet	3: score ?	No score ?	?	Poss	No	No	No	2
[89]	Primary	1: Ecz	No 5%: Pet (geranium)	1: Score ?	No score ?	Geranium	Poss	No	No	Lip balm	3

Note: Primary = primary report of cases; Multi. Cent. = multicenter study, may be reported separately; Review = primary reports cited; CosmA = cosmetic allergic; Ecz = eczematic, Frag A = fragrance allergic; FM + = fragrance mix positive; Dose, V = ? = dose and vehicle unknown; Pet = petrolatum; Acet = acetone; EtOH = ethanol; Score ? = intensity of reaction not recorded; + = mild, erythema only; ++ to +++ = more severe; irr = irritation; Immediate = immediate reactions, possibly irritant; 10 in 5 = 10 substances produce reactions in five patients; 2–3/patient = two to three substances on average produce reactions per patient; Sens ? = not clear if it is allergic contact dermatitis; Poss = cross-reactions are possible but cannot be determined from the report; Phased testing = procedures to avoid "excited skin" producing false positives; and Coinc. = coincidence of reaction to substance and to cosmetic product. However, no indication that substance is in product.

99 Operational Definition of a Causative Contact Allergen—Study with Six Fragrance Allergens

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Contact allergic dermatitis remains a significant public health problem. Its diagnosis and prevention is complicated by the difficulty of identifying allergens responsible for a patient's condition (i.e., those that have actually caused the allergic contact dermatitis). This chapter attempts to provide criteria that can be used as an operational definition of causative allergens. Six fragrance allergens exemplify the application of these criteria. Each is discussed in a separate paper (Hostynek, 2004; Hostynek and Maibach, 2003a, b; 2004a, b, c).

Although predictive tests can identify potential allergens, it is only through clinical diagnosis studies on patients with current contact dermatitis that truly causative allergens can be identified. This is, however, not a simple matter and is complicated by practical difficulties inherent to the technique of patch testing and the physiological nature of type IV allergy and by other practical matters such as the available time and the willingness of patients to submit to prolonged studies.

Clinical patch testing remains a partially subjective field (Storrs, 1994). When a clear reaction is observed, it is not always certain that it has been due to a truly clinically relevant allergic response. Marks et al. (1998) suggested that

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more than 40% of 3000 patients with suspected allergic contact dermatitis were, in fact, suffering from irritant or some other form of nonallergic contact dermatitis. A recent study has shown that there was an association between erythematous reactions to some allergens and irritant reactions to sodium lauryl sulfate, which is a putative marker for hyperreactive skin, thus allowing many reactions of this type to be classified as irritant rather than allergic in nature (Geier et al., 2003).

Apart from false-positive reactions from irritancy, there is always a possibility that other false-positive reactions can occur from cross-reactions in which patients react to substances that are not the primary sensitizers that initially induce the allergic state (Benezra et al., 1985). Similarities in chemical structure or cutaneous metabolism would appear to be the major factors in cross-reactivity (Smith and Hotchkiss, 2001). Over 70 different cross-reacting pairs or families of fragrance ingredients have been catalogued (Nater and de Groot, 1983; de Groot et al., 1994). Positive correlations of concomitant reactions in different pairs of components of the fragrance mix have also been recorded in polyreactive patients (Johansen and Menné, 1995).

False-positive reactions can also arise from phenomena such as the "excited skin syndrome" that occurs after a number of patches result in positive test results, which cannot be reproduced when the patient is retested (Maibach et al., 1982; Mitchell and Maibach, 1997). Over 40% of such positive patch reactions are lost on repatching (Mitchell, 1975; Bruynzeel et al., 1983; Mitchell and Maibach, 1997). Indeed, some studies have involved phased patch testing schedules to avoid false positives due to this syndrome.

Even when there is clear evidence that the reaction is allergic in nature, ascertaining the clinical relevance of the patch test requires knowledge of technical aspects relating to specificity and sensitivity issues (Ale and Maibach, 1995). In one of the more familiar clinical correlations—nickel allergic contact dermatitis—there is a high ratio of false-positive and false-negative reactions (Kiefer, 1979). Even when an allergic reaction has been indicated, the chance that an experienced physician will accurately identify the causative allergen from clinical information is about 50% of the time, mostly when common allergens are involved, but this is reduced to 10% for less common allergens (Fischer et al., 1989).

There is increasing evidence that diagnostic patch testing may also elicit true allergies, but these allergies are not the cause of the patient's current contact dermatitis. Lachapelle (1997) has defined clinical relevance as "the capability of an information retrieval system to select and retrieve data appropriate to a patient's need." In this context, he has distinguished between past relevance (not directly related to the patient's current problems) and current relevance and has devised a system for distinguishing between the two. He also defines the need to determine the "intrinsic imputability" of a suspected allergen. He defines this as the "possible (and not necessarily exclusive) cause–effect relationship between each positive test to an allergen and the occurrence of a given chemical event." The approach proposed here uses some aspects of this system. A more recent publication (Lachapelle and Maibach, 2003) gives strategies for determining true clinical relevance. These strategies require establishing the existence of past exposure and ensuring that the patient's exposure has indeed been responsible for the observed dermatitis. A number of suggestions are also made for improving evidence-based diagnosis of relevance. These include running use tests with implicated products, accounting for possible cross-reactions and a more rigorous and detailed examination of the case in which the clinician must retrieve the pertinent historical data, trace the responsible environmental exposure, and perform the appropriate tests.

Although some allergies revealed by patch testing may pertain to past allergic clinical events, others may pertain to allergies that have been acquired by the patient but that have never been clinically manifested (i.e., have never caused contact dermatitis in the patient; Boukhman and Maibach, 2001). There is increasing experimental evidence mainly in animal tests (Chan et al., 1983; Jayjock and Lewis, 1992; Nakamura et al., 1999; Yamano et al., 2001; van Och et al., 2001; Scott et al., 2002) and also in some older studies on humans (Friedmann and Moss, 1985) to show that the threshold dose of elicitation varies in accordance with the conditions of induction. When induction conditions are severe, the elicitation threshold dose is low. When induction occurs under mild conditions, much higher exposures are required to elicit an allergic reaction. This means that it may be possible for patients to have acquired allergies under low-exposure conditions (e.g., from using cosmetics or other consumer products resulting in relatively mild exposures to their allergenic components) that will never be elicited during their everyday lives as long as exposure remains low. However, these allergies may be artificially elicited under higher exposure conditions experienced in patch testing. These true-positive reactions may not be clinically relevant and indeed may not represent a cause for concern for the patient as they reflect an allergic state that may never manifest itself clinically.

Diagnostic patch tests are by necessity purposefully designed to avoid false negatives (i.e., to avoid missing possible causative agents), and to do this, the patch-test conditions are intentionally more severe than normal exposure conditions. We have clarified that the patch-test dose (single application) is usually higher than the use test dose (Andersen et al., 2001; Johansen et al., 2003). This can be seen by comparing relative doses. Taking fragrance allergens as example, a fragrance ingredient used at 1% in a perfume spray (the product type that produces the highest on-skin level of fragrance), a maximum dermal loading of 26 $\mu g/cm^2$ is obtained (Gerberick et al., 2001). (NB: This would correspond to a fragrance ingredient present at 20% in a fragrance used at 5% in this type of product.) Yet the use of diagnostic patch tests with 1% of the same ingredient in 19 mm Hill Top Chambers® will deliver a skin loading of 1770 $\mu g/cm^2$ (Robinson et al. 2000), a 68-fold increase. The use of 2×2 cm^2 Webril® patches, 8 mm Finn Chambers®, and a Professional Products® 1.9×1.9 cm^2 patch would result in 38-, 11-, and 21-fold increases, respectively. To this, we should add the (dose-enhancing) potentiating

effects of occlusion (numerous publications including Kraus et al., 1990; Calvin and Menné, 1993; Ale and Maibach, 1995; Funk and Maibach, 1994; Zhai and Maibach, 2001) and the potentiation due to an exceptionally long duration of the 48-h exposure (McFadden et al., 1998). The intentions behind this type of exaggeration are laudable for being aimed at identifying substances to which the patient may be allergic. However, they will not necessarily identify the substance that is primarily responsible for the allergy from which the patient is suffering at that specific time. We will return to this particular point under Section 99.2.4.

Defining the appropriate concentrations for patch tests requires finding a balance between the need to avoid concentrations that are high enough to cause irritation and active sensitization yet provide an appropriate enhancement of normal use concentrations to permit identification of allergic individuals.

The situation with fragrance allergens remains complex because of the relatively limited data on which appropriate patch-test concentrations can be defined and, until recently, the relative difficulty in obtaining documentation regarding the individual fragrance ingredient and its concentration in a given consumer product. However, the International Fragrance Association (IFRA, 2003) has recently carried out a number of industry-wide surveys, which have been aimed at determining the highest concentrations currently used in fine fragrance products (i.e., the type of consumer products that deliver the highest levels of fragrance in terms of concentration and quantity per unit area). On the basis of these levels, we propose in the Annexure the concentrations that could be used as guides to determine concentrations that could be used in some common patch-test systems. These concentrations would correspond approximately to the maximum exposure that could be expected from using a consumer product when the dermatologist is confronted with a case of allergic contact dermatitis but has no culprit product to examine.

Depending on whether a response is irritant or allergic has long been a complex challenge. All too frequently, inadequate (nonallergic) controls are available. Excited skin syndrome (Maibach, 1981) provides further complexity; most clinicians do little single-patch tests to verify this possibility. Brasch (Brasch and Henseler, 1992) and Geier (Geier et al., 2003) have suggested using their reaction index/positives ratio as a retrospective aid in defining the allergens whose positive responses might, in fact, be irritant instead.

In the ideal, but rarely encountered, clinical situation, the causative role of an allergen will be suggested by its presence in a consumer product that has already been identified as the cause of the patient's allergy. In such cases, the patch test should be conducted at a concentration that is related to the concentration of the suspected allergen in the product.

We suggest in Sections 99.1 and 99.2 the criteria by which the causative role of an allergen can be attributed to a specific case of clinical contact dermatitis. By far, the more important is diagnostic patch testing as this alone can link the substance to the case. The criteria are aimed at determining the degree of confidence we can ascribe through diagnostic patch testing, a specific allergen's causal role in a specific case of allergic contact dermatitis. It is also important, however, to have some measure of the substance's intrinsic allergenic potency. For this reason, we are also presenting a scheme for ascribing a degree of confidence to results of predictive tests.

These schemes build on a previous approach of Benezra et al. (1985), and in subsequent papers; these criteria are put into practice by taking six fragrance allergens recently identified as major fragrance allergens (SCCNFP, 1999; European Commission, 2003). These have been specifically chosen to span the range of likely causality shown by the 24 allergenic substances identified in this new legislation. Two of the substances are considered as frequently encountered allergens (geraniol, amylcinnamic aldehyde), two are among the less frequently encountered (α-iso-methylionone, anisyl alcohol), and two are intermediate in this regard (citronellol, linalool).

99.1 DATA OBTAINED FROM DIAGNOSTIC PATCH TESTING OF PATIENTS IN DERMATOLOGICAL CLINICS

The following scoring system is proposed:

99.1.1 Is It a Primary Case Report or a General Review?

Many publications in this area are reviews or statistical studies of patch-test results already published elsewhere. Although these papers perform an important function, they present a possible source of duplicate reporting whereby the same patch-test result is referred to twice in the literature. It is therefore important to distinguish between these two types of study. A higher degree of confidence is attributed to detailed primary studies.

99.1.2 Is Information Provided on the Number and Condition of the Patients?

The number of patients being examined is of importance mainly for epidemiological studies. However, when studies do not provide information on the number and nature of the test materials to which individual patients reacted, it is impossible to estimate the degree of polyreactivity and possible cross-reactions. It is also important to know if the patients suffer from current eczema or from other diseases. A lower degree of confidence is attributed when this information is not provided.

99.1.3 Are the Conditions of Patch Testing Given?

This is of primary importance particularly with regard to the purity of the test material (Fregert, 1989). The test material should be clearly identified and the degree of purity specified. The presence of potentially more allergenic impurities (e.g., aldehydes in alcohols, autoxidation products) should

also be controlled and indicated. A lower degree of confidence is attributed to results from studies in which such verifications are not reported.

Ideally, the report should give a detailed description of the patch-test conditions. The type and size of the patch, duration of occlusion, nature of the vehicle, and the concentration of the test material are minimum informations that are required. The bioavailability of the test material and the dose in quantity per unit area will vary from one type patch-test kit to another (Fischer and Maibach, 1990; Robinson et al., 2000). The timing of readings is also of importance.

Unless it is clear from historical evidence, it is necessary to rule out the possibility that irritant reactions are observed. For this reason, it has been recommended to undertake a preliminary test of at least three concentrations on control subjects (Fischer and Maibach, 1990).

A higher degree of confidence is also attributed when it is clear that the patch-test conditions have exposed the patient to levels of the allergen that are not disproportionately high with regard to the levels of exposure that were suspected to have led to the patient's condition. Although there is no clear test to determine whether a positive patch-test reaction has revealed the cause of the allergy or some latent subclinical allergy, information on the conditions of patch testing provides valuable information in making a judgment with any degree of confidence in this regard.

99.1.4 Are the Results Reported in Sufficient Detail?

The intensity of positive reactions should be recorded. Numerous authors have expressed concern that a significant proportion of patch-test reactions may be irritant in nature. This is particularly the case with weakly positive (1+) scores (Fisher, 1980), and it has been proposed that these scores should be handled separately (Lachapelle et al., 1988). Studies comparing TRUE Test™ and Finn Chambers showed that the fragrance mix gave about 47% irritant and questionable reactions with the former and about 45% with the latter, with a high degree of discordance between the two systems. In other large studies (Schnuch et al., 1997; Uter et al., 1998), about 60–70% of the reactions recorded were 1+ and it was speculated that up to 40% were irritant in nature (Schnuch et al., 1997). Further, the fact that the skin is not viewed for 24 h following the application of the patch makes it almost impossible to distinguish between quick-developing irritation and delayed contact hypersensitivity.

Scores should be given in a way that allows comparison of reactions experienced by a given patient to different test materials. A higher degree of confidence is attributed to results from studies in which this information is provided.

99.1.5 Is It Possible to Ascertain if Patients Had Reacted to Other Materials?

Concomitant reactions cloud the issue of causality. Although it is possible that a particular case of allergic contact dermatitis has been caused by several allergens, other explanations for multiple reactions to different suspect allergens will need to be ruled out. For this reason, it is important to know what other substances produced positive patch-test reactions in each patient.

99.1.6 Can We Rule out Cross-Reactions?

A lower degree of confidence is attributed to results from studies in which uncertainty arises in this regard. When positive reactions occur to different substances in the same patient, and these substances have similar chemical structures, the possibility arises that some of these patch-test reactions are in fact false indicators of the true cause of the case of contact allergic dermatitis.

99.1.7 Can We Rule out "Excited Skin"?

There is also the possibility that false-positive reactions appear due to the "excited skin syndrome" (Maibach et al., 1982; Mitchell and Maibach, 1997; Mitchell, 1975; Bruynzeel et al., 1983). The likelihood of this occurring can be reduced by carrying out patch tests in a time-phased manner so that the number of patches is minimized. A higher degree of confidence is allocated to studies that have taken such precautions or that have taken other measures to ensure that some of the observed reactions are not due to artifacts of this type.

99.1.8 Has the Substance Been Tested in Usage Tests?

Although some uncertainties in these techniques need to be resolved (Nakada et al., 2000), the use of repeat open applied tests (ROATs) (provocative use tests [PUTs]) can add important extra evidence of the causative role as they confirm under milder conditions than patch testing the allergenic role of the substance. If these can be performed on suspected sources of exposure to the patient (e.g., consumer products containing the substance), an even higher degree of confidence is attained (see Section 99.1.9). This is a key to the scheme proposed by Lachapelle (1997) for assigning "intrinsic imputability."

99.1.9 Have the Substance and the Allergy Been Linked to a Specific Consumer Product or Exposure Situation?

This is perhaps the gold standard for establishing the causal responsibility of a suspected allergen. Ideally, this would go further than simply gathering information of the possibly causative products (the third criteria of Lachapelle, 1997) and would include carrying out patch tests on fractions of the(se) causative product(s) until the culpable allergens have been identified by producing reactions to the exclusion of all other components. A number of studies producing convincing results of this type have been published (for example, Handley and Burrows, 1994). However, in such cases, the highest degree of confidence can only be ascribed when it is

clear that the patch-test conditions have exposed the patient to levels of the allergen that are not disproportionately high with regard to the levels of exposure that were suspected to have led to the patient's condition.

The following scoring system has been used:

5	Meets all criteria
4	Meets all criteria, but number of cases is marginal
3	Meets criteria, but some parameters are questionable
2	Evidence does not unambiguously indicate causative role of the test substance
1	Fails several criteria; results are not considered to be reliable
0	Fails all criteria/not primary report in literature of cases cited

99.2 DATA OBTAINED FROM PREDICTIVE TESTS IN ANIMALS AND HUMAN VOLUNTEERS

The first part of this analysis examines the likelihood that the designated substance shows an inherent potential to sensitize. This type of information is best obtained from predictive tests, although it should be cautioned that these data only provide information on a substance's intrinsic hazard. The conditions of exposure to the general public may be sufficiently high in some instances to produce reactions in substances that fail to show any significant sensitization hazard (Menné et al., 2002). For this reason, the apparent absence of a sensitization potential from predictive tests will not rule out the likelihood that a substance will not cause reactions in sufficiently exposed populations. Given this proviso, we suggest that the degree of confidence should be attributed to predictive tests according to the following criteria.

99.2.1 Was the Test Material Clearly Identified?

The test material should be clearly identified, and the degree of purity should be specified. The presence of potentially more allergenic impurities (e.g., aldehydic impurities in alcohols, autoxidation products) should also be controlled. A lower degree of confidence is attributed to results from studies in which such verifications are not reported.

99.2.2 The Type of Test/Type of Test Subjects

The type of test should be clearly specified as should the nature of the test subjects. If these are human subjects, their dermatological status should have been verified by a dermatologist prior to and, if necessary, during the course of the study. It is also accepted that some types of tests are more sensitive than others. Adjuvant tests in guinea pigs have long been regarded as more sensitive than nonadjuvant methods. Some tests have been less well validated than others. A lower degree of confidence is attributed to results from studies that are not as sensitive as others.

99.2.3 Were Details of Test Conditions Provided?

Information on the exact protocols should be provided to ensure that the most sensitive methodology has been applied. The test concentration (and the skin loading as expressed in quantity per unit area) should be neat to the limit of irritation. The choice of the vehicle(s) and the type of patches can also have an influence on the sensitivity of the test. A lower degree of confidence is attributed to results from studies in which this information is not reported or in which it is deemed less than optimal. Officially approved test protocols should be used for those methods that have been codified in this way (e.g., by OECD, 1981 as amended). There is also accumulating evidence to show that occlusion and ethanol may in fact produce false positives in human studies. This should also be taken into account.

99.2.4 Were These Sufficiently Maximized?

A lower degree of confidence is attributed to results from studies that are deemed to be less than optimally maximized to avoid false-negative results.

99.2.5 Were There Adequate Controls?

Control subjects are necessary to ensure that irritancy is not occurring during induction. Tests involving a challenge phase should include challenges to naive subjects to control irritancy. Where ethically possible, the laboratory performing these studies should carry out regular positive control studies using standard borderline allergens. A lower degree of confidence is attributed to results from studies in which these controls are not reported to have been used or in which they have also produced reactions.

99.2.6 Was the Number of Test Subjects Sufficient?

There are international standards requiring the minimum number of animals to be used in some tests (OECD, 1981 as amended). Tests on human subjects should generally involve more than these due to the inherent and environmental variability of the test subjects (Henderson and Riley, 1948), and ideally at least 200 should be used. A lower degree of confidence is attributed to results from studies in which an insufficient number of test subjects were used.

99.2.7 Were the Results Presented in Sufficient Detail?

The intensity of positive reactions should be recorded. Scores should also be followed for individual test subjects to ensure that, for instance, subjects reacting at one challenge are the same as those reacting at subsequent challenges. A lower degree of confidence is attributed to results from studies in which this information is not provided.

99.2.7.1 Scoring

The following scoring system is used:

5	Meets all criteria
4	Meets all criteria, but number of positives is marginal
3	Meets criteria, but some parameters are questionable (e.g., insufficient data provided or test not fully maximized)
2	Controls apparently absent or small number of test subjects
1	Fails several criteria; results are not considered to be reliable
0	Fails all criteria

99.3 CONCLUSIONS OF THE STUDIES ON SIX FRAGRANCE ALLERGENS

The accompanying papers on amylcinnamic aldehyde (Hostynek, 2004), anisyl alcohol (Hostynek and Maibach, 2003a), citronellol (Hostynek and Maibach, 2004b), geraniol (Hostynek and Maibach, 2004c), linalool (Hostynek and Maibach, 2003b), and α-iso-methylionone (Hostynek and Maibach, 2004a) show that when the underlying clinical and experimental data are analyzed according to the criteria outlined in parts 99.1 and 99.2, a clear cause–effect relationship has infrequently or rarely been established, nor would one be necessarily expected on the basis of the generally weak sensitizing potential of these substances coupled with reasonably low-exposure conditions. This is not to say that some of these substances are frequent inducers of type IV allergy in members of the public. It remains to be seen, however, that how often such allergy, once established, is responsible for any of the cases of allergic contact dermatitis commonly ascribed to these substances.

Schnuch and others have commented extensively on the wisdom of and criteria for the definition of a chemical allergen in man. Marrakchi and Maibach (1994), Ale and Maibach (2000), and Schnuch et al. (2002) provide a state of the science.

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ANNEXURE

Patch-Test Concentrations That Correspond to Those Experienced in Maximum Consumer Exposure

	Maximum Exposure from Cosmetics		Equivalent Concentrations in Standard Patch-Test Kits[c]			
Test Material	**Concentration[a] (%)**	**Quantity/ Unit Area[b] (μg/cm²)**	**Finn Chambers (8 mm) (%)**	**Hill Top Chambers (19 mm) (%)**	**Professional Products (1.9 × 1.9 cm) (%)**	**Webril (2 × 2 cm) (%)**
Amylcinnamic aldehyde	0.89	23	0.08	0.013	0.04	0.023
Anisyl alcohol	0.57	15	0.05	0.008	0.03	0.015
Citronellol	0.70	18	0.06	0.01	0.03	0.018
Geraniol	0.62	16	0.05	0.009	0.03	0.06
Linalool	0.86	22	0.075	0.013	0.04	0.022
α-iso-Methylionone	0.74	19	0.064	0.011	0.035	0.019

Note: Finn Chambers (8 mm): 30 mg/cm², Hill Top Chambers (19 mm): 177 mg/cm², Professional Products patch (1.9 × 1.9 cm²): 55.4 mg/cm², Webril patch (2 × 2 cm): 100 mg/cm².

[a] Data used by the research institute for fragrance materials (RIFM) from IFRA surveys; assumes 20% of fragrance in the cosmetic (fine fragrance product).

[b] From Gerberick et al. (2001); spray-on fragrance product delivers a maximum of 2.6 mg product/cm².

[c] Does not take into account of additional effects of occlusion and 48-h duration of patch tests; based on data from Robinson et al. (2000).

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100 Sodium Lauryl Sulfate-Induced Irritation in Human Faces: Regional- and Age-Related Differences

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100.1 INTRODUCTION

Although extensively studied [1], sodium lauryl sulfate (SLS) has been rarely used on the face to investigate mechanisms of irritation [2]. To our knowledge, no studies involving different areas of the human face have been published.

Because of the particular skin sensitivity of the face and the neck and because of the regional- and age-related variabilities detected in these areas to compounds inducing contact urticaria [3,4], we conducted this study with SLS 2% under occlusion for 1 h.

Because baseline trans epidermal water loss (TEWL) has been speculated as a predictive parameter to skin susceptibility to SLS [5] and changes in hydration of superficial epidermis suspected to be responsible for the seasonal variability of skin irritation induced by SLS [6], we measured the baseline TEWL and capacitance before SLS application and studied their correlation with changes in TEWL (∂TEWL), 1 and 23 h after patch removal [1].

100.2 MATERIAL AND METHODS

100.2.1 Subjects

Two age groups were examined: 10 young subjects, aged 25.2 ± 4.7 years ranging from 19 to 30 and 10 older subjects aged 73.7 ± 3.9 years ranging from 70 to 81.

All the volunteers gave their written consent and the study was approved by the local ethical committee.

100.2.2 Methods

Eight areas of the skin (forehead, nose, cheek, nasolabial and perioral areas, chin, neck, and volar forearm) were studied.

After 15 min of rest, necessary to suppress excess water evaporation, baseline TEWL was measured using an evaporimeter, Tewameter TM 210* (Courage + Khazaka, Cologne, Germany) and baseline capacitance was measured with a Corneometer CM 820 PC (Courage + Khazaka, Cologne, Germany).

Sodium lauryl sulfate (Sigma, St. Louis, MO) 2% (w/v) in water was then applied to each of the eight areas for 1 h under occlusion using a saturated absorbent filter paper disc (0.8 cm diameter) in Finn Chamber aluminum discs (Epitest Ltd Oy, Finland). On the contralateral side, water was applied in the same conditions as control.

To assess skin irritation, TEWL was measured 1 and 23 h after patch removal.

Trans epidermal water loss values of the areas tested were corrected according to the changes in the control areas:

$$\text{TEWL} = \text{TEWL measured} - \partial\text{TEWL } H_2O$$

where TEWL measured is the measured TEWL in the tested area at 1 or 23 h and ∂TEWL H_2O = TEWL control – baseline TEWL H_2O, where TEWL control is the measured TEWL value in the control area at 1 or 23 h.

The skin reactivity to SLS was assessed by the changes in TEWL (∂TEWL = TEWL – baseline TEWL).

100.2.3 Statistical Analysis

To compare the skin reactivity (∂TEWL) of the regions within each group, the two-tailed Student *t*-test for paired data was used. The two-tailed Student *t*-test for unpaired data was used to compare the two age groups.

Simple linear regression and correlation analysis between basal TEWL and skin irritation (∂TEWL) and between baseline capacitance and ∂TEWL for each skin location combining the data of the two age groups was used. ∂TEWL was considered as the dependent variable.

100.3 RESULTS

100.3.1 Skin Reactivity

Sodium lauryl sulfate 2% under occlusion for 1 h induced in most of the cases a subclinical irritation and sometimes minimal erythema.

The absolute TEWL values taken after 1 and 23 h did not show significant differences. Since the 23-h measurements demonstrated lower standard deviation (SD) values, only the irritation assessed at 23 h will be considered.

100.3.1.1 Comparison Between the Regions

In the young group all the areas reacted to SLS except the forearm. Skin irritation induced by SLS and assessed by ∂TEWL was greater in the cheek and chin when compared to the neck and forearm ($p < .05$).

The highest ∂TEWL mean values were found in the cheek and chin (Table 100.1) but no statistically significant differences with the remaining regions of the face were detected.

All the other regions except the forehead showed a significantly higher irritation than the forearm.

In the old group, all regions reacted to SLS except the nose, perioral area, and forearm. The cheek and chin showed the highest ∂TEWL mean values (Table 100.1). Significantly ($p < .05$) higher reactivity of these two areas was found when compared to the forearm and when the chin is compared to the forehead.

100.3.1.2 Comparison Between the Two Age Groups

The mean ∂TEWL values were higher in all the areas studied in the young than in the older group (Table 100.1). Only in the chin ($p = .035$) and nasolabial area ($p = .005$) the differences were significant.

100.3.2 Correlation Study

100.3.2.1 Correlation Between Baseline TEWL and ∂TEWL

Table 100.2 summarizes the correlations in each area between baseline TEWL and ∂TEWL 23 h after patch removal.

TABLE 100.1
Reactivity of Regions in the Young and Old Group

	∂TEWL (Mean ± SD) g/m² h		
Area	**Young Group**	**Old Group**	***p* Value**
Cheek	15.1 ± 12.8	6.8 ± 7.3	0.093
Chin[a]	13.5 ± 9.9	6.0 ± 3.3	0.035
Forearm	1.9 ± 2.1	1.1 ± 1.5	0.354
Forehead	10.4 ± 13.9	2.3 ± 2.3	0.086
Neck	6.8 ± 6.0	3.6 ± 3.7	0.165
Nasolabial area[a]	12.4 ± 6.3	4.4 ± 4.8	0.005
Nose	8.6 ± 7.6	5.0 ± 6.0	0.251
Perioral area	10.7 ± 10.0	4.2 ± 4.1	0.074

Note: ∂TEWL = TEWL 23 h after patch-test removal corrected to the control—baseline TEWL.

[a] Difference between the young and old group statistically significant ($p < .05$).

Source: Marrakchi, S., and Maibach, H.I. in *Skin Pharmacology and Physiology*, Karger, Switzerland, 2006, vol. 19, 177–180. With permission.

TABLE 100.2
Correlations in Each Area Between Baseline TEWL (BTEWL) and Reactivity of the Skin to SLS, 23 h after Patch Removal (∂TEWL)

	BTEWL (Mean ± SD)	TEWL 23 h (Mean ± SD)	∂TEWL (Mean ± SD)	*r*	*p*
Cheek[a]	15.63 ± 6.70	26.63 ± 15.30	10.96 ± 11.01	.4616	.040
Chin[a]	20.87 ± 6.37	30.47 ± 12.08	9.77 ± 8.13	.3535	.126
Forearm	8.64 ± 3.97	9.70 ± 4.92	1.51 ± 1.83		
Forehead[a]	14.10 ± 5.71	20.40 ± 14.96	6.39 ± 10.53	.6474	.002
Neck[a]	11.55 ± 4.35	16.63 ± 8.54	5.18 ± 5.12	.6273	.003
Nasolabial[a]	28.74 ± 8.56	36.93 ± 13.44	8.40 ± 6.78	.4831	.031
Nose[a]	19.04 ± 6.03	25.27 ± 11.15	6.77 ± 6.92	.3218	.166
Perioral	24.25 ± 8.93	29.98 ± 14.6	7.47 ± 8.17	.4547	.044

Note: Areas which reacted to SLS: statistically significant ($p < .05$) difference baseline TEWL and TEWL 23 h after patch removal. r = coefficient of correlation. p = significance (significant correlation when $p < .05$).

[a] Difference between the young and old group statistically significant ($p < .05$).

Source: Marrakchi, S., and Maibach, H.I. in *Skin Pharmacology and Physiology*, Karger, Switzerland, 2006, vol. 19, 177–180. With permission.

The forehead and the neck showed the strongest correlations ($r = .6474$, $p = .002$ in the forehead; $r = .6273$, $p = .003$ in the neck).

The nose and chin did not demonstrate a significant correlation between basal TEWL and TEWL changes induced by SLS. The forearm was not studied since this area did not react to the surfactant.

100.3.2.2 Correlation Between Baseline Capacitance and ∂TEWL

The baseline capacitance was not correlated to the skin irritation induced by SLS in any area studied.

100.4 DISCUSSION

Sodium lauryl sulfate, an anionic surfactant is widely used to study the sensitivity of the skin to irritants. Little information on the susceptibility of the face to SLS is available [2].

In this study, we investigated the influence of age and regional variability on SLS irritation with a focus on the skin of the face.

We considered only TEWL 23 h after patch removal because of the lower SD when compared to the 1 h values. This difference in SD might be explained by the "transient damage to the water barrier of the skin" described by Agner and Serup [7] and induced by exposure to water. This transient increase of TEWL not related to SLS, or to the evaporation of additional water lasts between 1 and 3 h after patch removal.

Considering the increase of TEWL after SLS exposure (∂TEWL), the young group had a higher irritant response than the old group in the chin and nasolabial area. In the remaining regions including the neck, ∂TEWL mean values were higher in the young group although the differences were not significant. This lack of significant differences might be explained by the high SD values (Table 100.1) in these regions. Previous studies [2,8] investigated the influence of age on the susceptibility to SLS and reported a decrease of the sensitivity in the elderly, which is in concordance with our results.

Various protocols (concentrations, application time) use SLS in water solution to induce skin irritation [9,10]. In our study, since the face was suspected to be more sensitive than the remaining regions of the body, and for a practical purpose, SLS 2% was applied only for 1 h under occlusion. This protocol was sufficient to induce subclinical irritation in most of the areas of the face but not in the forearm confirming that the face is more sensitive than the forearm.

Although the cheek and chin showed the highest ∂TEWL mean values, no regional variations were detected between the various regions of the face in both age groups, but the cheek and chin were more sensitive than the neck in the young group. This lack of significant differences between regions might be explained by the high SD observed in ∂TEWL values.

To see whether significant differences in skin irritation induced by surfactant exist between the regions of the face, higher SLS concentrations should be tested as well as repeated open applications, which may better reflect the common use of potential irritants on the face.

The correlation study showed a significant correlation between basal TEWL and ∂TEWL in five of the seven areas that have reacted to SLS (Table 100.2).

The correlations between baseline TEWL and TEWL 23 h after patch removal were more obvious. All the areas that reacted to SLS (all the areas studied except the forearm) showed a strong correlation coefficient varying between 0.76 and 0.88, with a highly significant p value $< .001$.

However, we think that the correlation between basal TEWL and the absolute TEWL value after irritation does not imply that higher basal TEWL values predispose to higher skin sensitivity, but only the correlation between baseline TEWL and the changes in TEWL after irritation (∂TEWL)

may have this significance. Even if for different basal TEWL values, the changes in TEWL are the same, a positive correlation could be found because TEWL is considered as a stable parameter [11].

Conflicting results have been published with regard to this aspect. Some authors correlated the absolute TEWL values before and after irritation [5,12–14]. Others [15] used basal TEWL and changes in TEWL (∂TEWL). Agner [15], studying healthy and atopic subjects, reported a positive correlation between baseline TEWL and the increase in TEWL induced by SLS only in the healthy group. Although in the atopic group, basal TEWL was significantly higher than the normal subjects, the changes after SLS exposure were not significantly different between the two groups.

These findings are in concordance with our study where some areas of the face (nasolabial area) showed higher basal TEWL values than others (the cheek) but failed to demonstrate higher sensitivity (Table 100.2). So, each region of the face has probably its own characteristics influencing the skin sensitivity to irritants probably independent from basal TEWL.

Further studies are needed to detect the participation, with baseline TEWL, of biophysical, biochemical, or anatomical parameters in the susceptibility of the face to irritants.

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101 Classification of Irritant Contact Dermatitis

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101.1 INTRODUCTION

Contact dermatitis is defined as inflammation of the skin invoked as a result of exposure to an exogenous agent, and constitutes a key portion of occupational disorders in industrialized societies.

In 1898, contact dermatitis was first appreciated to have more than one mechanism, and is now generally divided into *irritant contact dermatitis* (ICD) and *allergic contact dermatitis* (ACD), on the basis of these mechanistic differences. ACD is a delayed (type IV) hypersensitivity reaction, mediated by T cells and requiring prior sensitization, whereas ICD has a nonimmunologic mechanism, thus not requiring sensitization. Clinical distinction of the two processes is often challenging, as morphology and histopathology of irritant and allergic dermatitis reactions can be virtually indistinguishable. The two processes may, and often do, coexist, thereby further complicating matters.

The morphological spectrum of ICD is broad and frequently impossible to distinguish from ACD and even endogenous (atopic) dermatitis. Chronological descriptions of these processes are often clinically used. Acute, subacute, and chronic dermatitis are terms applicable to ACD and ICD, as well as atopic dermatitis. The erythema, edema, and vesiculation seen in acute dermatitis, or the hyperkeratosis, lichenification, and fissuring seen in the chronic phase, are largely nonspecific signs. Although chronologic classification has its uses, the main classification of irritation is now based on both morphology and clinical course of the dermatitis.

101.2 CLINICAL CLASSIFICATION OF ICD

ICD (synonyms: *cutaneous irritation*, *irritant dermatitis*) is the biological response of the skin to a variety of external stimuli that induce skin inflammation without the production of specific antibodies. Formerly considered a monomorphous process, it is now understood to be a complex biologic syndrome, with a diverse clinical appearance, pathophysiology, and natural history. The clinical appearance and course of ICD varies depending on multiple external and internal factors. This diversity in clinical presentation has generated a classification scheme, on the basis of both morphology and mode of onset. The various "types" of ICD and their respective prognoses are tabulated in Table 101.1.

101.2.1 Acute ICD

When exposure is sufficient and the offending agent is potent, classic signs of acute skin irritation are seen. Erythema, edema, inflammation, and vesiculation are typical features, although acute irritation may range from mild erythema through exudative cutaneous inflammation to ulcerative lesions and frank epidermal necrosis, depending on factors such as the chemical and the exposure time.[1] At the extreme

TABLE 101.1
Ten Genotypes of ICD

Irritation	Onset	Prognosis
1. Acute ICD	Acute—often single exposure	Good
2. Delayed acute ICD	Delayed—12–24 h or longer	Good
3. Irritant reaction	Acute—often multiple exposures	Good
4. Chronic ICD	Slowly developing (weeks to years)	Variable
5. Traumatic ICD	Slowly developing after preceding trauma	Variable
6. Acneiform ICD	Moderately slowly developing (weeks to months)	Variable
7. Nonerythematous (suberythematous) irritation	Slowly developing	Variable
8. Subjective (sensory) irritation	Acute	Excellent
9. Friction dermatitis	Slowly developing	Variable
10. Asteatotic irritant eczema	Slowly developing	Variable

end of this spectrum is the "chemical burn"—this entity is recognized by severe tissue damage as a result of exposure to highly alkaline or acidic compounds—most often as a result of an industrial accident. Symptoms of acute ICD are pruritus, burning, stinging, and pain.

In keeping with an exogenous dermatosis, acute ICD usually exhibits an asymmetrical distribution and sharply demarcated borders. These borders delineate the area of exposure to the offending chemical. Contact with a potent irritant is often accidental, and an acute ICD is elicited in almost anyone, independent of constitutional susceptibility—in contrast to chronic ICD.

This classic, acutely developing dermatitis usually heals soon after exposure, assuming there is no reexposure—this is known as the "decrescendo phenomenon." In contrast, ACD usually exhibits a "crescendo phenomenon," i.e., transient worsening of symptoms and signs despite removal of the allergen. In unusual cases, ICD may persist for months after exposure, followed by complete resolution.

The availability of the material safety data sheet and data from the single-application Draize rabbit test combined with activities of industrial hygienists and other informed personnel have greatly decreased the frequency of such dermatitis in industry.

101.2.2 Delayed Acute ICD

Some chemicals produce acute irritation in a delayed manner so that inflammation is retarded until 8–24 h or more after exposure.[2] Except for the delayed onset, the clinical appearance and course resemble those of acute ICD. The delayed acute irritant dermatitis, because of its delayed onset and atypical "crescendo" periodicity, is often confused with ACD; appropriately performed diagnostic patch tests easily separate the two, i.e., the substances implicated in delayed, acute ICD would result in negative patch-test results. In delayed acute ICD, a burning sensation predominates, rather than pruritus. Examples of substances causing delayed irritation are hexanediol and butanediol diacrylates,[2] dithranol (anthralin), calcipotriol, and benzalkonium chloride.

101.2.3 Irritant Reaction

Individuals extensively exposed to irritants often develop erythematous, chapped skin in the first few months of exposure. This irritant reaction may be considered a preeczematous expression of acute skin irritation. The term "irritant reaction" is now increasingly used if the clinical picture is monomorphic, rather than the usual polymorphic appearance of ICD, i.e., only one of the parameters usually seen in ICD is present, e.g., scaling, erythema, vesiculation, pustules, or erosions. This pattern is frequently seen in hairdressers and other wet workers. Frequently, this condition heals spontaneously, with hardening of the skin. However, repeated irritant reactions can sometimes lead to contact dermatitis, usually with good prognosis. Compounds that cause irritant reactions are typically mild irritants, such as detergents, soaps, and water.

101.2.4 Chronic ICD

When exposure inducing an acute irritant dermatitis is repeated, the dermatitis tends to persist and becomes chronic (more than 6 weeks has been suggested as an arbitrary threshold period). In chronic ICD (synonyms: *cumulative ICD*, *traumiterative dermatitis*, *wear and tear dermatitis*), the frequency of exposure is too high in relation to the skin recovery time.

Multiple subthreshold skin insults lead to a manifest dermatitis when the irritant load exceeds the individual's elicitation threshold for visible effects. Chronic ICD was called "traumiterative dermatitis" in the older German literature ("traumiterative" = traumas repeating).[3,4] Classic signs are erythema and increasing xerosis (dryness), followed by hyperkeratosis with frequent fissuring and occasional erythema. The lesions are usually localized but ill defined. Pruritus and pain due to fissures are symptoms of chronic ICD. Chronic ICD often presents as hand eczema ("housewives' eczema").

Chronic ICD is the most common type of ICD. This clinical picture may develop after days, weeks, or years of subtle exposure to chemical substances. Variation in individual susceptibility and the physical properties of the irritating substance increase the multiplicity of clinical findings.

Delayed onset and variable attack lead to confusion with ACD. To rule out an allergic etiology, appropriate diagnostic patch testing is indicated. Models of chronic ICD have been developed, contributing to product evaluation and mechanistic insights.[5,6]

101.2.5 Traumatic ICD

Traumatic ICD develops after acute skin trauma, such as burns, lacerations, or acute ICD. The skin does not completely heal, but erythema, vesicles, papules, and scaling appear at the site of injury. The clinical course later resembles discoid (nummular) dermatitis. It may be compounded by a concurrent allergen exposure. The healing period is generally prolonged.

Often these patients are considered to have factitial dermatitis because of a healing phase followed by exacerbation. Although factitial aspects may occur in some patients, this peculiar form of irritation appears to be a disease *sui generis*. Its chronicity and recalcitrance to therapy provides a challenge to both patient and physician.

101.2.6 Acneiform ICD

Certain exogenous substances have the capacity to elicit an acneiform eruption,[7,8] and even allergic reactions may sometimes be pustular or follicular.[9] Acneiform ICD (synonyms: *pustular ICD*, *follicular ICD*) should always be considered in the differential diagnosis of an adult with acneiform lesions. The pustules are usually sterile and transient.

In occupational exposure, only a minority of subjects develop pustular or acneiform dermatitis. Thus, the development of this type of ICD appears to be dependent on both constitutional and chemical factors. *Chloracne* is an industrial disease caused by exposure to chlorinated aromatic hydrocarbons, in particular chlorinated dioxins, which are the most potent acnegenic agents. Many of the chloracnegens are also hepatotoxic—therefore this is a disease of medical importance. Acneiform ICD may also develop from exposure to metals, mineral oils, greases, tar, asphalt, cutting oils, and metal working fluids.

Acne cosmetica represents acneiform ICD caused by cosmetics. *Pomade acne* is a well-known form of acne cosmetica, seen in Afro-Caribbean women who apply vegetable oils to their skin.[10] A similar problem has been reported with applications of white petrolatum.[11] Nowadays, most cosmetics available in Western countries are noncomedogenic and nonacnegenic.

101.2.7 Nonerythematous or Suberythematous Irritation

In the early stages of skin irritation, subtle skin damage may occur without visible inflammation. As a correlate of nonvisible irritation, objectively registered alterations in the damaged epidermis have been reported via cutaneous bioengineering techniques.[12–14] It is customary in Japan to screen new chemicals, cosmetics, and textiles for subtle signs of stratum corneum damage, employing replicas of stratum corneum (the Kawai method[22]). A similar technique, squamometry or corneosurfametry, has now been refined to detect subtle subclinical alterations in the stratum corneum caused by application of mild irritants.[15]

101.2.8 Subjective or Sensory Irritation

Some individuals ("stingers") experience itching, stinging, burning, or tingling sensations on contact with certain chemicals,[14,16] despite a distinct lack of objective signs on clinical examination. Despite the lack of clinical manifestations, the subjective sensations are reproducible, typically occurring within seconds to minutes following exposure; this type of irritation is known as subjective or sensory irritation. Lactic acid is a model for this nonvisible cutaneous irritation. The threshold for this reaction varies between subjects, independent of susceptibility to other irritation types. The quality as well as the concentration of the exposing agent is also important, and neural pathways may be contributory, but the pathomechanism is unknown. Some sensory irritation may be subclinical contact urticaria. Screening raw ingredients and final formulations in the guinea pig ear swelling test[17] or the human forehead assay allows us to minimize the amount of subclinical contact urticaria.

Although subjective irritation may have a neural component, recent studies suggest that cutaneous vasculature may be more responsive in "stingers" than nonstingers.[14,18] At least 10% of women complain of stinging with certain facial products; thus, further work is needed to develop a strategy to overcome this type of discomfort.

101.2.9 Friction Dermatitis

Repeated friction of low intensity is known to induce callus formation (hyperkeratosis and acanthosis), hardening of the skin, hyperpigmentation, and friction blisters in normal skin. In atopic people, lichenification and lichen simplex chronicus may ensue friction. All of the above may be considered as adaptive phenomena to friction and should not be confused with friction dermatitis.

True friction dermatitis is the development of ICD in response to low-grade friction—this is seen clinically as erythema, scaling, fissuring, and itching around the area of frictional contact. This syndrome has been characterized by Susten.[19] Cases of occupational friction dermatitis in the literature are seldom documented, but most often reported in association with paper work.[20] More recently, a short collection of further cases of friction dermatitis has been published.[21]

101.2.10 Asteatotic Irritant Eczema

Asteatotic eczema (synonyms: *asteatotic dermatitis*, *exsiccation eczematid*, *eczema craquele*) is a variant of ICD seen in elderly individuals, as a result of worsening xerosis, particularly during dry winter months. Clinically, the skin is dry (xerosis), with loss of smoothness, ichthyosi-

form scale, and cracking of the superficial epidermal layers, often associated with eczematous changes. The term "eczema cracquele" refers to the cracked, patchy eczematous appearance (like cracked porcelain, or "crazy paving"), usually seen on the lower legs of these individuals. An uncomfortable sensation of "tightness" and pruritus is often felt.

Xerosis is a result of low water content in the stratum corneum, causing the stratum corneum to lose its suppleness and the corneocytes to be shed in large polygonal scales. Xerosis is usually more pronounced in the elderly and in atopic individuals. Environmental insults, such as low humidity, low temperatures, and very high doses of ultraviolet radiation (UVR) (>3 or 4 minimal erythema dose (MED's)) can help accelerate this process. In an occupational setting, this is sometimes combined with repeated exposure to wet work, chemical insults, and friction, cumulating in perturbation of the skin barrier. Skin barrier dysfunction then leaves the skin even more vulnerable to exogenous insults and asteatotic irritant eczema ensues.

101.2.11 Miscellaneous

Airborne ICD is not included in this classification scheme as the mechanisms are similar to acute or chronic ICD—the only difference is that the irritant substance is dispersed and transported in the air before contact with skin. This causes dermatitis on exposed areas of skin, most commonly on the face and may mimic photoallergic reactions.

Phototoxicity or *photoirritation* is another form of skin irritation following cutaneous or systemic exposure to a phototoxic agent in combination with appropriate radiation (most often in the ultraviolet A (UVA) spectrum). *Phytophotodermatitis* specifically represents phototoxic dermatitis in response to plants or plant derivatives, such as species in the Umbelliferae (e.g., celery, carrot) and Rutaceae (e.g., lime, lemon, bergamot) families. *Berloque dermatitis* refers to fragrance dermatitis due to bergapten, the photoactive compound found in oil of bergamot, an ingredient found in fragrances—this compound has now been removed from most perfumes and substituted with artificial or highly refined bergamot oil.

Other reactions that can be caused by contact with irritant substances, but do not fall within the scope of this chapter, include pigmentary alterations, nonimmunologic contact urticaria, granulomatous reactions, and alopecia.

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102 *In Vitro* Skin Irritation Testing on SkinEthic™-Reconstituted Human Epidermis: Reproducibility for Fifty Chemicals Tested with Two Protocols

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102.1 INTRODUCTION

Evaluation of the irritancy potential to human skin of any chemical or formulation used in the chemical, pharmaceutical, and cosmetics industries is a necessity. Several *in vivo* and *in vitro* tests aim to determine the risk of irritation resulting from the contact between these compounds and human skin. The most commonly used test is the rabbit skin irritation test described in the OECD test guideline 404 and in the European Chemicals Bureau Annex V part B.4 (http://ecb.jrc.it/testing-methods/) and initially described by Draize et al. (1944). This animal test consists in applying topically substances on the rabbit's shaved skin, which are raw materials or formulations (i.e., finished products). A score is attributed according to physiological observations on the animals, which allows the classification of each tested product.

However, the Draize test presents several major disadvantages. The first is due to the fact that rabbit skin and human skin have different physiological properties and responses to environmental and chemical agents (Phillips et al., 1972; Marzulli and Maibach, 1975; Nixon et al., 1975; Scott et al., 1991). Unfortunately the biological basis for the variability of skin irritation among species remains unknown (Campbell and Bruce, 1981). However, rabbit data were taken as reference to determine the irritant potential of chemicals although, to the exception of rare publications (Phillips et al., 1972; Nixon et al., 1975), few studies have compared data obtained on both animals and humans. Some compounds are more toxic for rabbits than for humans and vice versa

(Phillips et al., 1972; Nixon et al., 1975; ECETOC, 2002). Moreover, the Draize test lacks reproducibility (Weil and Scala, 1971; Spielmann, 1996). The third major inconvenience concerns animal's suffering and discomfort since eschar formation can be observed with severe irritants.

Few experiments were done on humans because of direct risk of lesion and intoxication for the subject resulting from the application of potentially dangerous compounds. Among the available human data on toxicity of chemicals, some derives from chemical insults with severe irritants due to accidents at home or at work, or due to repeated skin exposure to moderate irritants. The other human data for skin irritation testing were obtained by patch testing performed on relatively high numbers of volunteers (Finkelstein et al., 1965; Nixon et al., 1975; Smiles and Pollack, 1977; Piérard et al., 1994; Effendy and Maibach, 1995; Basketter et al., 1997, 1999; Zhai and Maibach, 2004). Compounds were tested pure or diluted, for different application times, but the experiments were stopped when moderate-to-severe reactions to the test compound were observed. Many parameters influence the reproducibility of this type of tests. First, variability is observed in function of the patches used. York et al. (1995) showed that generally the "Webril" and the "Hill Top" patches produced greater reactivity than the "Van der Bend" and the "Finn" patches. Some other parameters responsible for the variability of test results are directly correlated with the choice of the volunteers: the interindividual variability of reactivity is the principal factor (Basketter et al., 1996). Moreover, interethnic differences have been observed (Foy et al., 2001; Robinson, 2002). The reactivity of human skin also changes with the anatomical site (Lee and Maibach, 1995), and decreases with age (Robinson, 2002). Even abiotic factors must be considered since the seasonal variability plays a role in skin reactivity (Robinson et al., 1998; Geier et al., 2003). The seasonal effect was particularly evident in the experiment described by Basketter et al. (1996) when a 4 h patch test with SDS 20% provoked skin irritation in 45% of the volunteers in summer, but increased to 91% in winter.

The development of *in vitro* alternative methods for testing skin irritation has been the aim of an increasing number of scientists. This can be explained by their ethical advantage, and in several cases, also by their enhanced convenience. The skin irritation function test (SIFT) (Heylings et al., 2003) and the pig ear test (Fentem et al., 2001) are two of these *in vitro* methods. These tests are thus performed on *ex vivo* animal tissues (mouse and pig, respectively).

To permit the testing on human tissues without the disadvantages of performing tests directly on humans, the development of cell and tissue culture appeared promising. Several models are now commercially available for testing skin irritation (Rougier et al., 1994; Rosdy et al., 1997; de Brugerolle de Fraissinette et al., 1999; Chew and Maibach, 2000; Faller et al., 2002). Using reconstructed epidermis, all classical methods determining cell viability (such as MTT reduction, resazurin reduction, LDH release, etc.) are easy to perform. Moreover, the SkinEthic™ model allows the measurement of additional endpoints such as the release of IL-1α and IL-8 (Doucet et al., 1996; de Brugerolle de Fraissinette et al., 1999; Coquette et al., 2003; Wells and Schröder, 2003). Reconstructed epidermis consists of human epidermal cells cultured in chemically defined medium by using semiautomatic production procedures, producing human epidermis standardized in terms of thickness, terminal differentiation, and reactivity to test compounds.

The repeated experiments described here are performed on different batches of human reconstructed epidermis. They correspond to different production cycles using epidermal cells of different donors. *In vitro* experimentation allows testing compounds on human tissues whatever the age, the gender, and the race of the donor. Each of the 50 chemicals has, at least, been tested in triplicate in two different experiments using two protocols: an *in vitro* patch test and a direct topical application test. The *in vitro* patch-test protocol mimics closely the human *in vivo* patch-test protocol (Basketter et al., 1997). We applied the compounds on 0.95 cm² polypropylene Hill Top (Cincinnati, OH, USA) chambers for 4 h. The quantity of chemicals applied is proportional to the size of the patches (0.95 cm²) used. This technique allows the containment of the product on a determined surface in the center of the 4 cm² epidermis. In parallel, our direct topical application test is performed by applying 100 µL of the test compound directly onto the epidermal surface of 0.63 cm² for 4 h. Among the 50 chosen chemicals, 20 chemicals were previously tested in the ECVAM prevalidation study on acute skin irritation (Fentem et al., 2001) (PVS chemicals), and 30 chemicals were previously tested in the *in vivo* human patch test described by Basketter et al. (1997) (HPT chemicals). After test compound application, tissues were incubated at 37°C, 5% CO_2 for 4 h in both protocols. The 20 PVS chemicals were tested in two additional separate experiments using the direct topical application protocol (four times in total). Multiple endpoint analysis including cell viability (MTT reduction), histology, and IL-1α release measurements was performed. Absence of direct interaction between test chemicals and the MTT solution or nonspecifically on frozen-killed tissues was verified. Our goal was to study the reproducibility of reference chemical testing on epidermis with two convenient protocols, and to compare the results with available *in vitro*, as well as animal and human *in vivo* data.

102.2 MATERIALS AND METHODS

102.2.1 Reconstituted Human Epidermis

Tissues (SkinEthic, Nice) used were fully differentiated 3D-reconstituted human epidermal cultures grown on the air–liquid interface for 17 days in defined growth medium (Rosdy and Clauss, 1990; Rosdy et al., 1997). Each experiment was performed in triplicate on one single tissue production batch, but different batches (different production cycles and donors cells) were used for each repeated experiment.

102.2.2 Selection and Coding of Test Chemicals

We chose reference chemicals upon two criteria: their irritation status should have been defined in the European Community

classification, and, furthermore, they should have been tested either on other three-dimensional models (Fentem et al., 2001) or by the human *in vivo* patch test (Basketter et al., 1997). The present study includes irritant and nonirritant compounds. Details of the 50 chemicals tested are presented in Table 102.1. The experiment performed on run B was realized as a blind test. The 20 HPT chemicals were coded by Maibach, UCSF, USA.

102.2.3 *In Vitro* Direct Topical Test Protocol

Three reconstituted epidermal tissues of 0.63 cm² on 0.3 mL defined maintenance medium in a 24-well plate were used per control or tested compound. Hundred microliters or 100 mg of test compounds were homogeneously displayed on the total surface of the reconstructed epidermis. Negative controls and positive controls were run in parallel for each experiment. Cultures were incubated for 4 h at 37°C, 5% CO_2. The three cultures were then transferred into new wells of the same 24-well plate containing 0.3 mL of maintenance medium. Tissues were washed three times with 0.5 mL saline solution A. With solids (powders or crystals), the insert was turned upside down before washing, and—maintained in this position with forceps—knocked two- to threefold on the inner wall of a beaker to mechanically remove most of the applied compound. Histology, MTT reduction, and IL-1α release endpoints were measured as described below. Untreated tissues and H_2O-treated tissues were used as negative controls while SDS 20% (Basketter et al., 1997; Fentem et al., 2001) and nonanoic acid (Wahlberg and Maibach, 1980) treated tissues were used as positive controls. Negative controls were considered satisfactory if three criteria were met: a high cell viability measured by MTT reduction ($\geq$ 85% of untreated epidermis), a normal histology (score $\geq$ 75) (see histology scoring below), and no release of large amounts of IL-1α ($<$ 30 pg/mL). Positive controls were considered satisfactory when a low cell viability was measured by MTT reduction ($<$ 50%), and when a necrosed histology (score $<$ 75) and an increase in the amount of secreted IL-1α ($\geq$ 30 pg/mL) were observed.

102.2.4 *In Vitro* Patch-Test Protocol

Three reconstituted epidermal tissues of 4 cm², placed on 1 mL defined maintenance medium in a 6-well plate, were used per control or test compound. Seventy-five microliters of the compound was homogeneously displayed on a 0.95 cm^2 Hill Top chamber (Cincinnati, OH, USA), which was immediately applied, carefully, to the center of a 4 cm² culture. In case of solid compounds, 75 mg of the powder or crystals was spread on 0.95 cm² (same surface as for liquids) on the center of the culture, and covered immediately by a Hill Top chamber. A 5 mm large brush was used to improve the contact between the compound/patch and the epidermal tissue. The patches were homogeneously applied with delicacy; strong pressure was avoided. Negative controls and positive controls were performed in parallel for each experiment. The Hill Top chamber was removed after a 4 h incubation at 37°C, 5% CO_2. No washing step was included in this protocol because most liquid compounds were absorbed by the patch. With solids, the culture was turned upside down, and—maintained in this position with forceps—knocked two- to threefold on the inner wall of a beaker to mechanically remove most of the applied compound. Histology, MTT reduction, and IL-1α release endpoints were performed as described below. Untreated tissues and H_2O-treated tissues were used as negative controls while SDS 20% (Basketter et al., 1997; Fentem et al., 2001) and nonanoic acid (Wahlberg and Maibach, 1980) treated tissues were used as positive controls. Negative controls were considered satisfactory if three criteria were met: a high cell viability measured by MTT reduction ($\geq$85% of untreated epidermis), a normal histology ($\geq$75) (see histology scoring below), and no release of large amounts of IL-1α ($<$105 pg/mL). Positive controls were considered satisfactory when a low cell viability was measured by MTT reduction ($<$50%), and when a necrosed histology ($<$75) and an increase in the amount of secreted IL-1α ($\geq$105 pg/mL) were observed.

102.2.5 Histology

Per test condition, and whatever the protocol, one of three tissues was harvested for histology. The tissues were fixed in a balanced 10% formalin solution and embedded in paraffin. Four microns vertical sections were stained with hematoxylin/eosin and photographed under a microscope.

Scoring of histology sections was performed as follows:

- 100: No or minor epidermal changes
- 75: Slight epidermal changes (stratum corneum thickening or dissociation or parakeratosis; slight edema or cellular alterations in the viable layers)
- 25: Severe epidermal changes (marked edema or less viable cell layers or cellular alterations or partial tissue necrosis or partial tissue disintegration)
- 0: Total tissue necrosis or tissue disintegration

102.2.6 Cell Viability Measurement by MTT Reduction

The MTT test was used to measure the viability of living cells via mitochondrial dehydrogenase activity (Mosmann, 1983). The ring of 3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), yellow in color, is cleaved by dehydrogenases, yielding blue/purple MTT crystals, which are insoluble in culture medium. An intense purple color is observed when the tissue is healthy, while the culture remains white when necrosis occurred.

Per test condition, the two remaining tissues were incubated in a 0.5 mg/mL MTT solution (0.3 mL MTT for 0.63 cm² cultures, and 1 mL MTT for 4 cm² cultures) for a 3 h incubation at 37°C, 5% CO_2. MTT crystals of 0.63 cm² inserts were dissolved in 2 mL isopropanol. In the case of the 4 cm² inserts, a 0.5 cm² biopsy from the treated center of the

TABLE 102.1
Chemicals Tested and Corresponding Skin Irritation Data

Compound	CAS no.	Supplier	EU Classification	OECD Classification	Human Patch Classification	S/L
1 Sodium lauryl sulfate (50%)	151-21-3	Sigma	I[a]	I[a]		L
2 1,1,1-Trichloroethane	71-55-6	Aldrich	I[a]	I[a]		L
3 Potassium hydroxide (5%)	1310-58-3	JT Baker	I[a]	I[a]		L
4 Heptanal	111-71-7	Aldrich	I[a]	I[a]		L
5 Methyl palmitate	112-39-0	Aldrich	I[a]/NC[b]	I[a]	NC[b]	L
6 Lilestralis/lilial	80-54-6	Aroma & Fine Chemical	I[a]	I[a]		L
7 1-Bromopentane	110-53-2	Aldrich	I[a]	I[a]		L
8 DL-Citronellol	106-22-9	Aldrich	I[a]	SLI[a]		L
9 D-Limonene	5989-27-5	Aldrich	I[a]	SLI[a]		L
10 10-Undecenoic acid	112-38-9	Aldrich	I[a]	SLI[a]		L
11 Dimethyl disulfide	624-92-0	Lancaster	NI[a]	SLI[a]		L
12 Soap from 20/80 coconut oil/tallow		Quimasso	NI[a]	NI[a]		S
13 *cis*-Cyclooctene	931-87-3	Aldrich	NI[a]	SLI[a]		L
14 2-Methyl-4-phenyl-2-butanol	103-05-9	Aldrich	NI[a]	NI[a]		L
15 2,4-Xylidine	95-68-1	Aldrich	NI[a]	NI[a]		L
16 Hydroxycitronellal	107-75-5	Astier-Demarest-Leroux	NI[a]	NI[a]		L
17 3,3′-Dithiodipropionic acid	1119-62-6	Aldrich	NI[a]	NI[a]		S
18 4,4-Methylene *bis*-(2,6-di-*tert*-butyl)phenol	118-82-1	Aldrich	NI[a]	NI[a]		S
19 4-Amino-1,2,4-triazole	584-13-4	Aldrich	NI[a]	NI[a]		S
20 3-Chloronitrobenzene	121-73-3	Aldrich	NI[a]	NI[a]		S
21 1-Decanol	112-30-1	Aldrich	R38[b]		NC[b]	L
22 2-Propanol	67-63-0	Aldrich	NC[b]		NC[b]	L
23 Isopropyl palmitate	142-91-6	Aldrich	NC[b]		NC[b]	L
24 Octanoic acid	124-07-2	Aldrich	R34[b]		R38[b]	L
25 Methyl caproate	106-70-7	Aldrich	NC[b]		NC[b]	L
26 Methyl laurate	111-82-0	Aldrich	R38[b]		NC[b]	L
27 Decanoic acid	334-48-5	Aldrich	R38[b]		R38[b]	L
28 Dodecanoic acid	143-07-7	Aldrich	R38[b]		NC[b]	L
29 *N*,*N*-Dimethyl-*N*-dodecyl aminobetaine 20%		Albright & Wilson	R38[b]		R38[b]	L
30 Benzalkonium chloride (10%)	8001-54-5	Sigma	R38[b]		R38[b]	L
31 Dimethyl sulfoxide	67-68-5	Sigma	NC[b]		R38[b]	L
32 Polyethylene glycol 400	25322-68-3	Aldrich	NC[b]		NC[b]	L
33 Acetic acid (10%)	64-19-7	Sigma	R38[b]		NC[b]	L
34 Hydrochloric acid (10%)	7647-01-0	Prolabo	R38[b]		NC[b]	L
35 Sodium hydroxide (0.5%)	1310-73-2	Sigma	R38[b]		R38[b]	L
36 Heptanoic acid	111-14-8	Aldrich	R34[b]		R38[b]	L
37 Lactic acid	50-21-5	Aldrich	NC[b]		R38[b]	L
38 Benzyl alcohol	100-51-6	Aldrich	NC[b]		NC[b]	L
39 Triethanolamine	102-71-6	Aldrich	NC[b]		NC[b]	L
40 Dodecanol	112-53-8	Aldrich	NC[b]		NC[b]	L
41 Tween 80	9005-65-6	Sigma	NC[b]		NC[b]	L
42 Benzalkonium chloride (7.5%)	8001-54-5	Sigma	R38[b]		R38[b]	L
43 Propylene glycol	4254-14-2	Fluka	NC[b]		NC[b]	L
44 Octanol	111-87-5	Aldrich	R38[b]		NC[b]	L
45 Eugenol	97-53-0	Sigma	R38[b]		NC[b]	L
46 Geraniol	106-24-1	Sigma	R38[b]		NC[b]	L
47 Linalyl acetate	115-95-7	Aldrich	R38[b]		NC[b]	L
48 Hexanol	111-27-3	Aldrich	R38[b]		NC[b]	L
49 α-Terpineol	10482-56-1	Aldrich	R38[b]		NC[b]	L
50 Ethanol	64-17-5	Merck	NC[b]		NC[b]	L

Note: I = irritant; NI = nonirritant; SLI = slight irritant; S = solid; and L = liquid.

[a] Fentem et al. (2001).

[b] Basketter et al. (1999).

culture was harvested using a 8 mm diameter biopsy punch (Stiefel), and plunged in 1 mL isopropanol. After an overnight extraction at room temperature, the quantification of cell viability was obtained by comparing the optical density of the extracts measured at 570 nm (reference filter 690 nm) in percentage to the negative H_2O-treated controls.

102.2.7 IL-1α Release

Conditioned media (three per test condition) underneath the epidermal cultures were collected after the 4 h incubation with the chemicals and kept frozen at –20°C. Inflammatory mediator IL-1α was measured quantitatively using ELISA kits (R&D Systems UK, Catalogue number DLA50) (Doucet et al., 1996; Coquette et al., 2003). Results were expressed in picograms of mediator released per milliliter of conditioned medium.

102.2.8 Direct Interaction Between MTT and Chemicals

Hundred microliters or 100 mg of each compound was incubated in 1 mL MTT solution (0.5 mg/mL) for 3 h at 37°C, 5% CO_2. The interaction was quantified by measuring the MTT/compound mixture OD value (200 μL in triplicate) at 570 nm (reference filter 690 nm).

102.2.9 MTT Interaction with Chemicals on Frozen-Killed Controls

The same procedure as for the direct topical application test protocol was applied onto frozen-killed tissues (−20°C, overnight). The results were expressed as the percentage of increase compared to the corresponding value of nontreated living control tissues.

102.2.10 Prediction Models

To classify the chemicals as irritants or nonirritants, we propose a prediction model based on the three endpoints described in sections 102.2.5, 102.2.6, and 102.2.7. A chemical was classified as nonirritant when two or three of the endpoints led to the following results: cell viability measured by MTT reduction over 50% compared to that of the H_2O-treated control, normal histology (score ≥75), and the release of IL-1α comparable to that observed for the H_2O-treated control (<30 pg/mL for the direct topical application test and ≥105 pg/mL for the *in vitro* patch test).

On the contrary, a chemical was classified as irritant since two or three of the endpoints measured correspond to the following criteria: cell viability lower than 50% compared to that of the H_2O-treated control, partial or total necrosis of the epidermal tissues (score <75), and an amount of secreted IL-1α higher than the IL-1α release induced by the H_2O-treated control (≥30 pg/mL for the direct topical application test and ≥105 pg/mL for the *in vitro* patch test).

In parallel, a single endpoint prediction model (viability by MTT reduction only) was used for comparison.

102.2.11 Statistical Analysis

Specificity corresponds to the percentage of nonirritant chemicals (according to the EU classification) identified as nonirritants in our test.

Sensitivity represents the percentage of irritant chemicals (according to the EU classification) identified as irritants in our test.

Accuracy corresponds to the overall percentage of correct classification.

Pearson correlations, slope, and variation coefficient were calculated by Dr. Els Adriaens (University of Gent, Belgium).

102.3 RESULTS

102.3.1 MTT Interaction with Chemicals

Most of the compounds did not present significant interaction with MTT, nor directly, nor on frozen-killed controls. However, these two additional control experiments have shown that the interaction was significant for three chemicals (eugenol, potassium hydroxide 5%, and heptanal) for at least one of the two experiments. First, a direct contact between eugenol and MTT solution quickly produced a dark blue/purple color (OD = 1.4) while, in parallel, the MTT solution alone remained yellow (OD = 0.0). Moreover, eugenol on frozen-killed epidermal tissues induced the change of color from white to dark blue/purple after 4 h incubation. The dissolution of the formazan blue crystals in isopropanol exhibited an OD value, which represented 82.2% of the OD value obtained for living untreated tissues (with the other 47 compounds, the epidermal tissues remained uncolored, and the OD values were negligible). Nevertheless, a strong direct interaction between a compound and MTT solution was not always correlated with an increased MTT value during the tests on living tissues or even on killed tissues. For example, potassium hydroxide 5% strongly reduced the MTT solution after direct contact, leading to a dark blue/purple mixture (OD = 1.6). On the contrary, potassium hydroxide 5% on frozen-killed tissues led to a light blue color of the culture. The corresponding OD value represented only 14.4% of the living untreated culture. Finally, in the case of heptanal, direct contact with the MTT solution provoked a change of color to blue (OD = 0.2). The application of heptanal on frozen-killed tissues also led to a blue color, with an OD value of 35% of the living untreated tissues.

102.3.2 *In Vitro* Direct Topical Test

Results of the multiple endpoint analysis of the repeated experiments are shown in Figure 102.1 (PVS chemicals) and Figure 102.2 (HPT chemicals). The percentage of cell viability (MTT reduction) is expressed in comparison to the H_2O-treated control. All controls of each experiment were satisfactory according to our criteria. Moreover, results obtained with the blind test were completely comparable to that of the normal test.

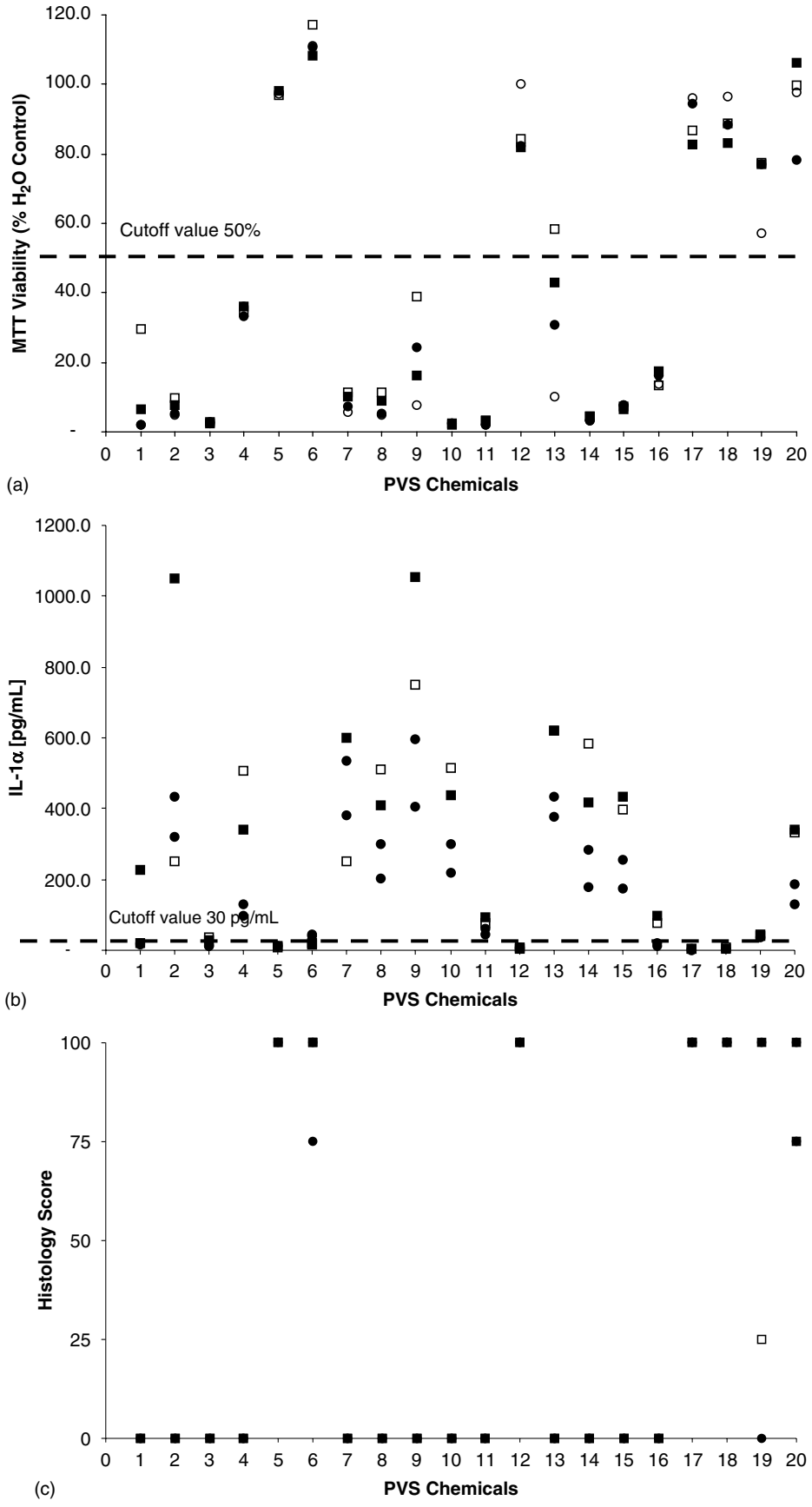
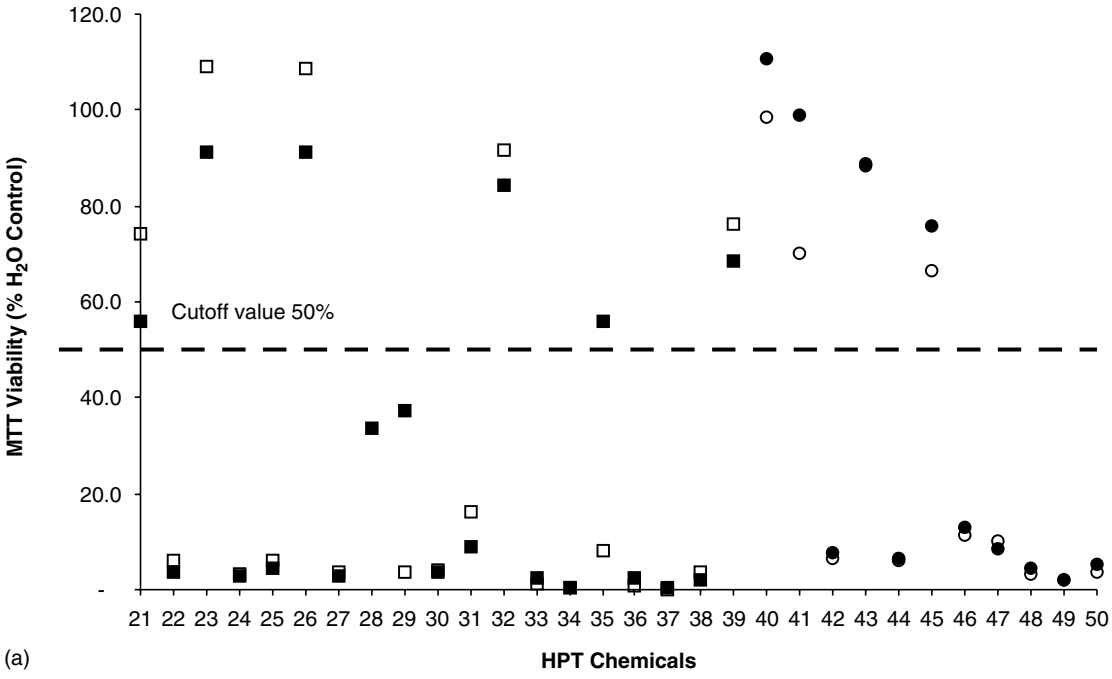
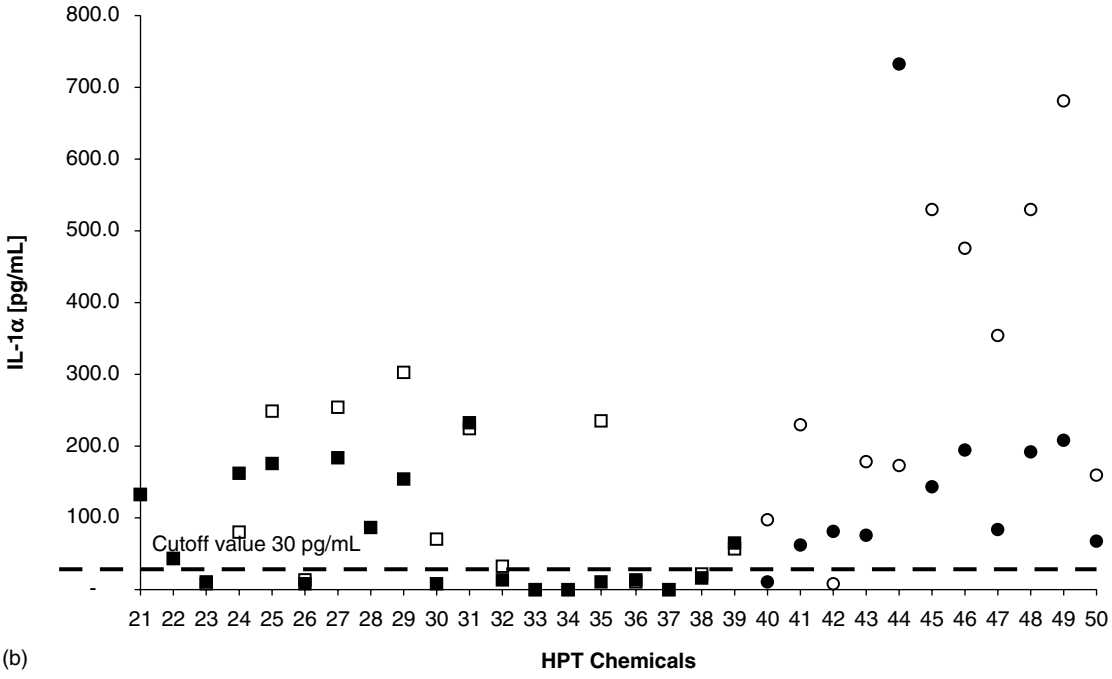


FIGURE 102.1 Multiple endpoint analysis for the PVS chemicals with the direct topical application test: (❑) run D, (■) run E, (○) run G, (●) run H. (a) Viability (MTT reduction assay). (b) IL-1α release. (c) Histological observations.

The response of the epidermal tissues to the chemicals could be classified in five families (Tables 102.2 and 102.3). The first is composed of compounds that induced a response comparable to that of the negative controls. The second family consists of the chemicals that allowed a high cell viability, a normal histology (score ≥ 75), but provoked an increase in IL-1α release (≥ 30 pg/mL). The third family is represented by chemicals that allowed a cell viability higher than 50%, but necrosis was visible on corresponding histological sections (score < 75), and an increase in the amount of IL-1α (≥ 30 pg/mL) release was measured. The fourth family includes chemicals that induced low cell viability and tissue necrosis (score < 75), but no release of large amounts of IL-1α (< 30 pg/mL). All remaining chemicals belong to the fifth and last family. This family is composed of chemicals that were responsible for a low tissue viability, tissue necrosis (score < 75), and a significant increase in secreted IL-1α (≥ 30 pg/mL) release. Whatever the family, we observed that in most cases the MTT values were of the same range for a given chemical. For example, among the 20 PVS chemicals for which four independent experiments were performed, dimethyl disulfide-induced MTT values contained between



(a)



(b)

FIGURE 102.2 *Continued*

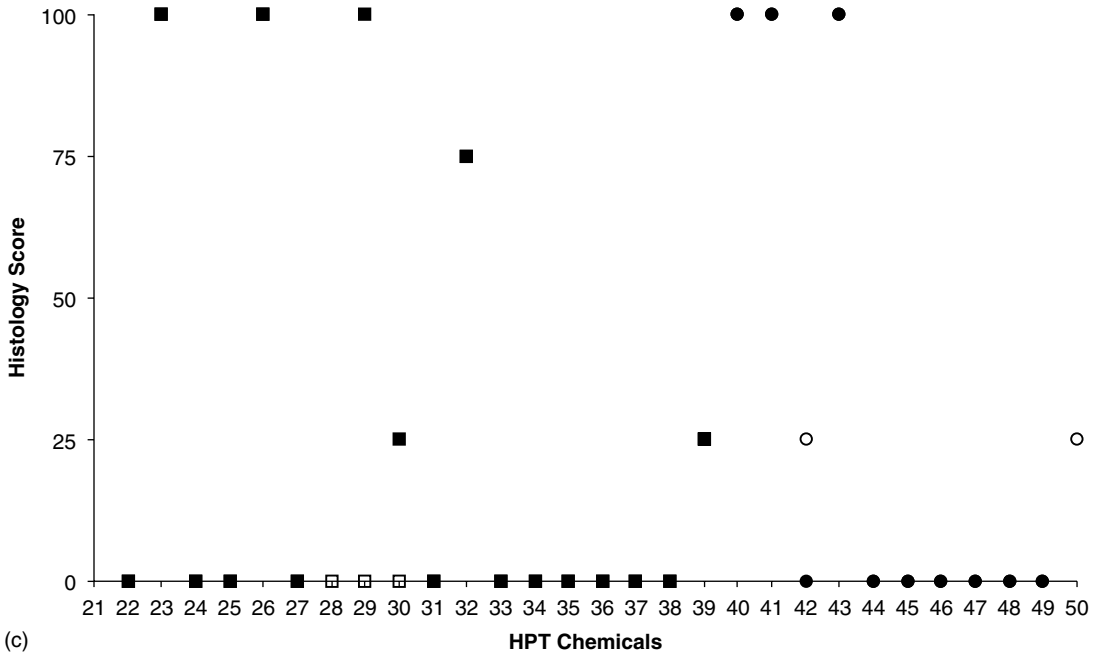


FIGURE 102.2 Multiple endpoint analysis for the HPT chemicals with the direct topical application test: (❑) run A, (■) run B, (○) run F, (●) run I. (a) Viability (MTT reduction assay). (b) IL-1α release. (c) Histological observations.

2.0 and 3.4% of negative control values; for heptanal they were contained between 33.1 and 36.0%, and for 3,3′-dithiodipropionic acid between 82.6 and 96.2%. Similarly, the histological appreciations were highly reproducible, especially when a given chemical was responsible for specific histological effects. Regarding the IL-1α release values, a reproducible effect was obtained. The chemicals induced two kinds of effects: On one hand, some compounds were responsible for an IL-1α release comparable to those of negative controls (e.g., methyl palmitate). On the other hand, some compounds provoked a significant increase in IL-1α release compared to negative controls (≥ 30 pg/mL), although the absolute values presented high variations. For example, D-limonene provoked large amounts of released IL-1α (404.2–1055.9 pg/mL), and these amounts were higher than those of the SDS 20% treated positive control tissues.

According to our prediction model based on multiple endpoint analysis, the first and the second families contained nonirritants; and the third, fourth, and fifth families contained irritants. The resulting classification is shown in Tables 102.2 and 102.3. A strong reproducibility was obtained between separate experiments. The one single exception for the 50 chemicals tested with this protocol was 4-amino-1,2,4-triazole (irritant in two and nonirritant in the other two experiments).

102.3.3 *In Vitro* Patch Test

Figure 102.3 (PVS chemicals) and Figure 102.4 (HPT chemicals) show the results obtained by multiple endpoint analysis with the *in vitro* patch test. As for the *in vitro* direct topical application test results (described above), both negative and positive controls were satisfactory. However, note that the amount of released IL-1α was four- to fivefold higher for the H_2O-treated control compared to that of the direct topical test. The ratio medium volume/tissue surface was 0.48 mL/cm² for 0.63 cm² tissues, compared to 0.25 mL/cm² for 4 cm² tissues. Also, the topical application of an empty patch induces a slight increase in basal IL-1α secretion. We thus considered that the level of released IL-1α has increased significantly when it was over 105 pg/mL. Moreover, the cell viability of the positive controls of the patch test was higher compared to those of the direct topical application test. More generally in this protocol, the percentage of cell viability was increased in most test conditions compared to the direct topical application protocol; this was probably due to the lower amounts (50%) of test chemicals, which, moreover, were applied to, and partially absorbed by, the patches.

The same classification in families as for the direct topical test was applied to the experiments performed with this patch-test protocol. However, a new sixth family has been created for hydroxycitronellal, 2-propanol, and hydrochloric acid 10%, which allowed a high cell viability, a small amount of IL-1α release, but provoked tissue necrosis as described in Section 102.2.5.

The same prediction model as for the direct topical test was applied to the patch-test results (Tables 102.2 and 102.3). A high intralaboratory reproducibility could also be observed with this protocol to the exception of *N*,*N*-dimethyl-*N*-dodecyl aminobetaine 20%, dimethyl sulfoxide, and dodecanoic acid, which were classified as irritant in one experiment and as nonirritant in the other.

TABLE 102.2
Comparison of the Results Obtained with the 20 ECVAM Chemicals

Compound	EU Classification	OECD Classification	EpiDerm Classification[a]	Episkin Classification[a]	SkinEthic Direct Application (Family) Classification	SkinEthic Patch (Family) Classification
1 Sodium lauryl sulfate (50%)	I[a]	I[a]	I	I	(4) I	(5) I
2 1,1,1-Trichloroethane	I[a]	I[a]	NI	I	(5) I	(5) I
3 Potassium hydroxide (5%)	I[a]	I[a]	I	I	(4) I	(5) I
4 Heptanal	I[a]	I[a]	I	I	(5) I	(5) I
5 Methyl palmitate	I[a]/NC[b]	I[a]	NI	NI	(1) NI	(1) NI
6 Lilestralis/lilial	I[a]	I[a]	I	I	(2) NI	(1) NI
7 1-Bromopentane	I[a]	I[a]	I	I	(5) I	(3) I
8 DL-Citronellol	I[a]	SLI[a]	I	I	(5) I	(3) I
9 D-Limonene	I[a]	SLI[a]	I	I	(5) I	(3) I
10 10-Undecenoic acid	I[a]	SLI[a]	NI	NI	(5) I	(5) I
11 Dimethyl disulfide	NI[a]	SLI[a]	I	I	(5) I	(4) I
12 Soap from 20/80 coconut oil/tallow	NI[a]	NI[a]	NI	NI	(1) NI	(1) NI
13 *cis*-Cyclooctene	NI[a]	SLI[a]	I	I	(5) I	(3) I
14 2-Methyl-4-phenyl-2-butanol	NI[a]	NI[a]	I	I	(5) I	(5) I
15 2,4-Xylidine	NI[a]	NI[a]	I	I	(5) I	(5) I
16 Hydroxycitronellal	NI[a]	NI[a]	I	I	(5) I	(6) NI
17 3,3′-Dithiodipropionic acid	NI[a]	NI[a]	NI	NI	(1) NI	(1) NI
18 4,4-Methylene *bis*-(2,6-di-*tert*-butyl)phenol	NI[a]	NI[a]	NI	NI	(1) NI	(1) NI
19 4-Amino-1,2,4-triazole	NI[a]	NI[a]	NI	NI	(3) NI/I	(2) NI
20 3-Chloronitrobenzene	NI[a]	NI[a]	I	I	(2) NI	(1) NI

Note: I = irritant; NI = nonirritant; SLI = slight irritant; and NC = nonclassified.
Family 1: high cell viability, normal histology, no release of large amounts of IL-1α release.
Family 2: high cell viability, normal histology, increase in the amount of IL-1α.
Family 3: high cell viability, necrosed histology, increase in the amount of IL-1α.
Family 4: low cell viability, necrosed histology, no release of large amounts of IL-1α.
Family 5: low cell viability, necrosed histology, increase in the amount of IL-1α.
Family 6: high cell viability, necrosed histology, no release of large amounts of IL-1α.
[a] Fentem et al. (2001).
[b] Basketter et al. (1999).

102.3.4 Comparison Between the Two Test Protocols

The comparison (Tables 102.2 and 102.3) between the predictions of the direct topical application test and those of the *in vitro* patch test shows that even when the experiments were performed using the two protocols, the final results were similar for most chemicals. However, the direct topical application test seemed more sensitive, since hydroxycitronellal, 2-propanol, *N*,*N*-dimethyl-*N*-dodecyl aminobetaine 20%, hydrochloric acid 10%, sodium hydroxide 0.5%, triethanolamine, and ethanol were detected as irritants with this protocol, although they appeared to be nonirritant with the *in vitro* patch test. These differences are discussed below.

Table 102.2 presents a summary of test results for the 20 PVS chemicals, including existing EU and OECD classifications, based on rabbit test results, as well as *in vitro* test results obtained with other tissue models (SkinEthic direct topical application test, SkinEthic *in vitro* patch test, Episkin™, and EpiDerm™ [Fentem et al., 2001]). All *in vitro* tissue models showed similar classifications of compounds whatever the tissue supplier. On the contrary, the comparison between the rabbit data and the human *in vitro* data revealed differences. In particular, this was true for the following compounds: dimethyl disulfide, *cis*-cyclooctene, 2-methyl-4-phenyl-2-butanol, and 2,4-xylidine. These compounds were classified as irritants by all *in vitro* tests, and as nonirritants or slightly irritants according to the EU and OECD classifications.

Hydroxycitronellal was the one single PVS compound with an opposite classification using our two protocols: in the *in vitro* patch test, it was found nonirritant, like in the European and OECD classifications, although it was classified as irritant with the SkinEthic direct topical application test, similarly to EpiDerm and Episkin classifications. Moreover, 4-amino-1,2,4-triazole (nonirritant in two experiments, and irritant in the two others, with the topical

TABLE 102.3
Comparison of the Results Obtained with the 30 Chemicals Tested in Human Patch Test

Compound	EU classification[a]	Human Patch Classification[a]	Skinethic Direct Application (Family) Classification	Skinethic Patch (Family) Classification
21 1-Decanol	R38	NC	(3) I	(3) I
22 2-Propanol	NC	NC	(5) I	(6) NI
23 Isopropyl palmitate	NC	NC	(1) NI	(1) NI
24 Octanoic acid	R34	R38	(5) I	(5) I
25 Methyl caproate	NC	NC	(5) I	(5) I
26 Methyl laurate	R38	NC	(1) NI	(2) NI
27 Decanoic acid	R38	R38	(5) I	(4) I
28 Dodecanoic acid	R38	NC	(5) I	(3/6) I
29 *N*,*N*-Dimethyl-*N*-dodecyl aminobetaine	R38	R38	(5) I	(1/3) NI/I
30 Benzalkonium chloride (10%)	R38	R38	(5) I	(5) I
31 Dimethyl sulfoxide	NC	R38	(5) I	(3/6) NI/I
32 Polyethylene glycol 400	NC	NC	(2) NI	(1) NI
33 Acetic acid (10%)	R38	NC	(4) I	(4) I
34 Hydrochloric acid (10%)	R38	NC	(4) I	(6) NI
35 Sodium hydroxide (0.5%)	R38	R38	(5) I	(1) NI
36 Heptanoic acid	R34	R38	(4) I	(5) I
37 Lactic acid	NC	R38	(4) I	(4) I
38 Benzyl alcohol	NC	NC	(4) I	(5) I
39 Triethanolamine	NC	NC	(3) I	(1) NI
40 Dodecanol	NC	NC	(2) NI	(1) NI
41 Tween 80	NC	NC	(2) NI	(1) NI
42 Benzalkonium chloride (7.5%)	R38	R38	(5) I	(5) I
43 Propylene glycol	NC	NC	(2) NI	(1) NI
44 Octanol	R38	NC	(5) I	(5) I
45 Eugenol	R38	NC	(3) I	(5) I
46 Geraniol	R38	NC	(5) I	(5) I
47 Linalyl acetate	R38	NC	(5) I	(3) I
48 Hexanol	R38	NC	(5) I	(5) I
49 α-Terpineol	R38	NC	(5) I	(5) I
50 Ethanol	NC	NC	(5) I	(1) NI

Note: R38/I = irritant; NC = nonclassified; R34 = corrosive; and NI = nonirritant.
Family 1: high cell viability, normal histology, no release of large amounts of IL-1α.
Family 2: high cell viability, normal histology, increase in the amount of IL-1α.
Family 3: high cell viability, necrosed histology, increase in the amount of IL-1α.
Family 4: low cell viability, necrosed histology, no release of large amounts of IL-1α.
Family 5: low cell viability, necrosed histology, increase in the amount of IL-1α.
Family 6: high cell viability, necrosed histology, no release of large amounts of IL-1α.
[a] Basketter et al. (1999).

application test) was classified unambiguously as nonirritant by the *in vitro* patch test.

A comparison between the EU classification, the human *in vivo* patch test, our *in vitro* patch test, and our *in vitro* direct topical application test is presented in Table 102.3. Among these 30 HPT chemicals, only 17 compounds were classified identically in the Draize test (EU classification) and in the human *in vivo* patch test (Basketter et al., 1999). Twelve of these seventeen chemicals were also classified similarly with the *in vitro* direct topical application test. Thirteen of these seventeen compounds were also classified similarly with the *in vitro* patch test. Ten of them are shared in common with the 12 cited above: isopropyl palmitate, octanoic acid, decanoic acid, benzalkonium chloride (10%), polyethylene glycol 400, heptanoic acid, dodecanol, Tween 80, benzalkonium chloride (7.5%), and propylene glycol. Among the seven other chemicals (of the 17), methyl caproate and benzyl alcohol were found irritant with both *in vitro* protocols, although they were classified as nonirritant by the EU and OECD classifications. For the five remaining compounds, the *in vitro* direct topical application test led to the most severe classification, although *N*,*N*-dimethyl-*N*-dodecyl aminobetaine 20% was classified

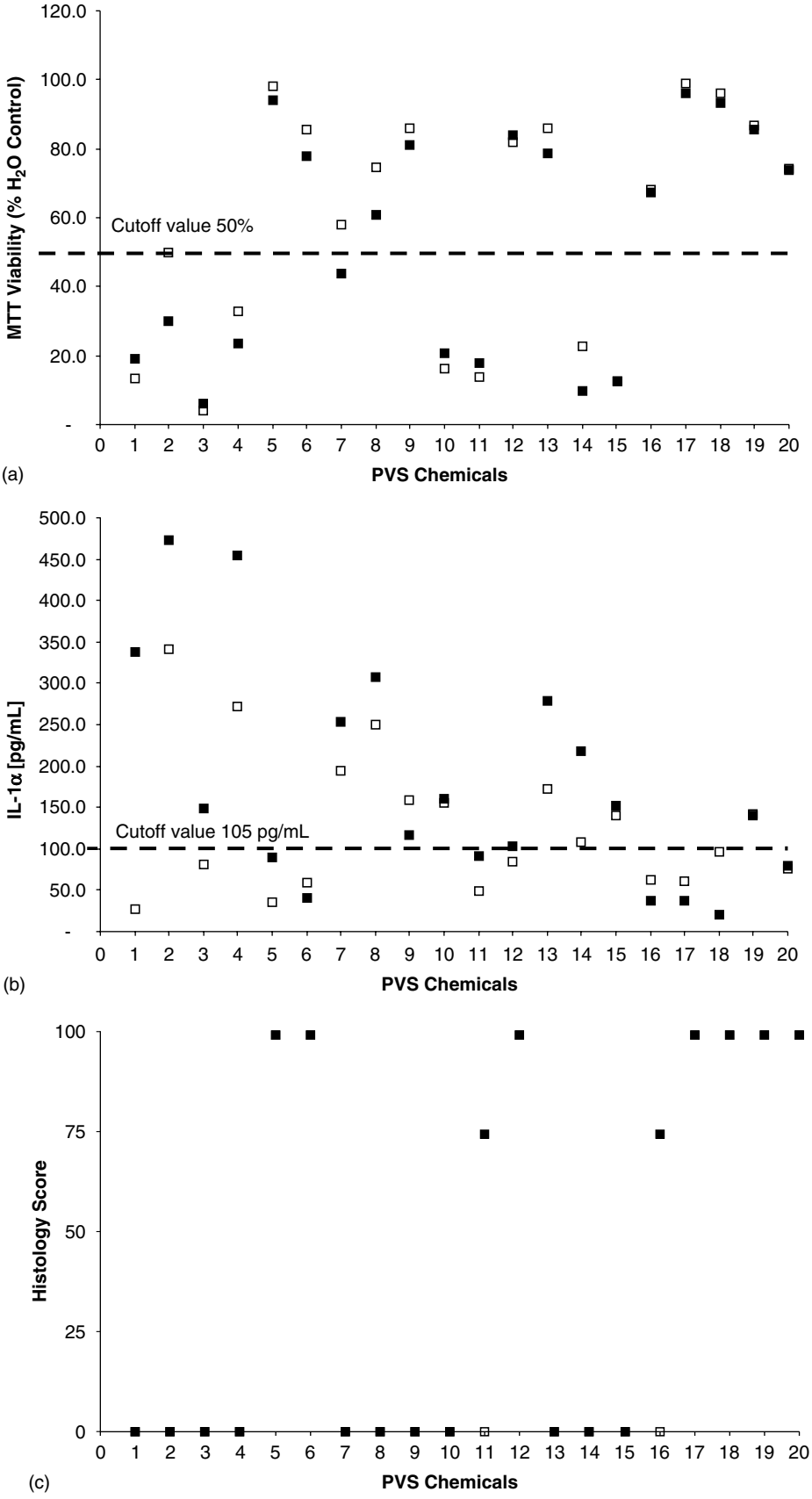


FIGURE 102.3 Multiple endpoint analysis for the PVS chemicals with the *in vitro* patch test: (❑) run D, (■) run E. (a) Viability (MTT reduction assay). (b) IL-1α release. (c) Histological observations.

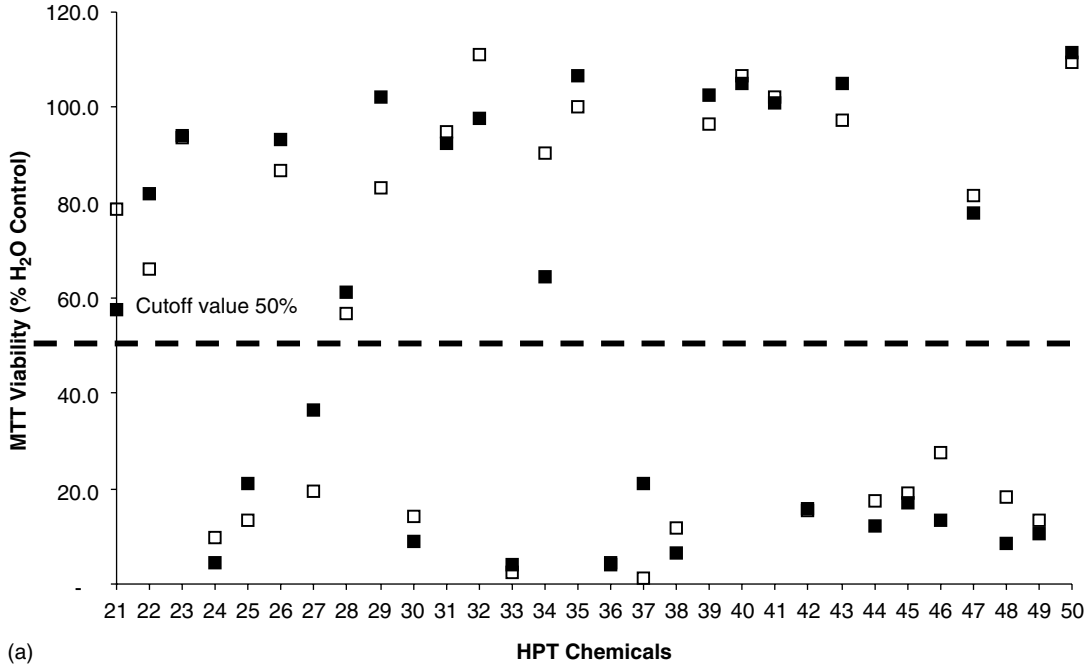
ambiguously, and 2-propanol, sodium hydroxide 0.5%, triethanolamine, and ethanol were classified as nonirritants with the *in vitro* patch test.

Among the 13 remaining chemicals, which were not classified similarly by the rabbit test and the human *in vivo* test, 12 compounds were found irritant with the *in vitro* direct topical application test. Thus, to the exception of the methyl laurate, which was classified as nonirritant, the *in vitro* topical application test also led to the most severe classification for these chemicals. In parallel, 10 of these 13 compounds were found irritant by the *in vitro* patch test; methyl laurate and hydrochloric acid 10% were classified as nonirritant, and dimethyl sulfoxide's classification was ambiguous.

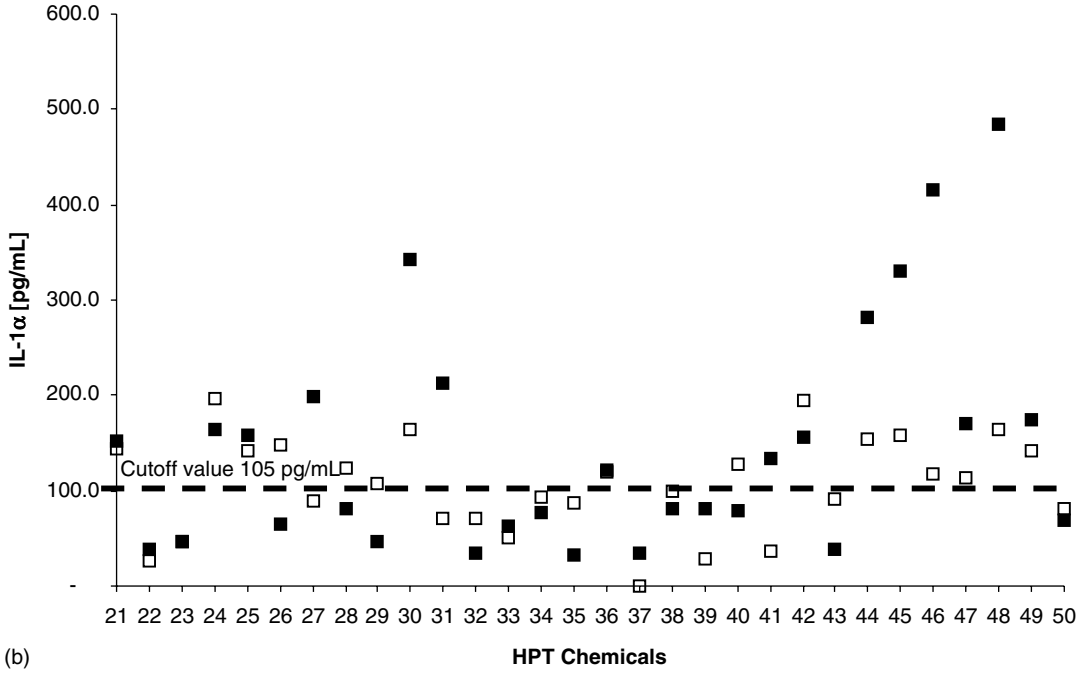
102.3.5 Statistical Reproducibility

When the classification of a tested compound was unclear (I/NI) the most pessimistic prediction (irritant) was chosen, according to the principle of precaution.

Statistical analysis (including Pearson correlation, slope, and variation coefficient) was performed for the different



(a)



(b)

FIGURE 102.4 *Continued*

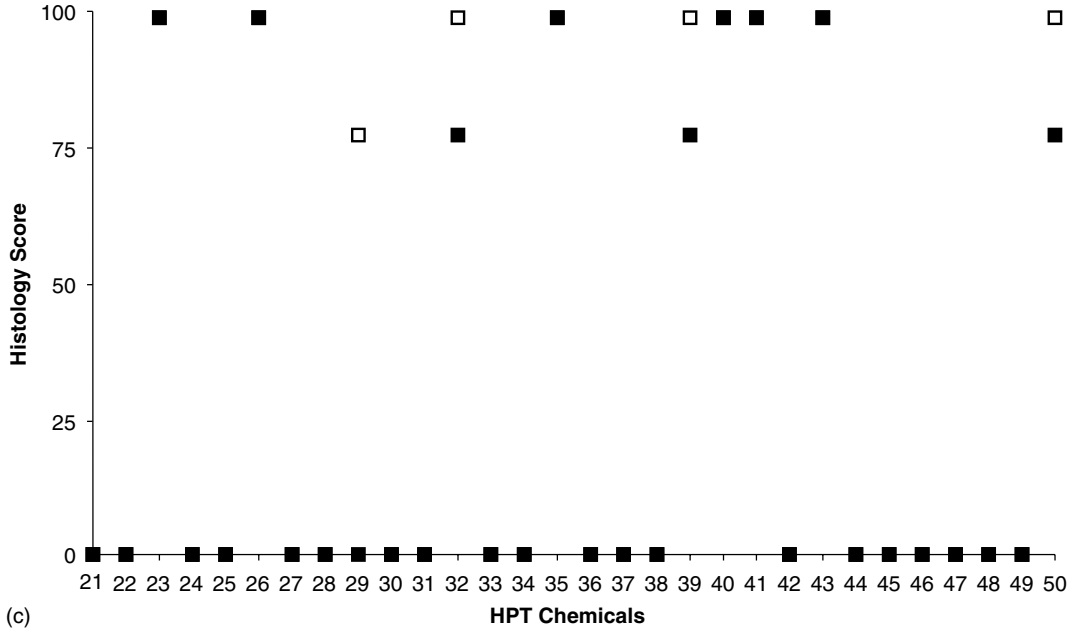


FIGURE 102.4 Multiple endpoint analysis for the HPT chemicals with the *in vitro* patch test: (❑) run C, (■) run J. (a) Viability (MTT reduction assay). (b) IL-1α release. (c) Histological observations.

endpoints of both the direct topical application test and the *in vitro* patch-test protocol. This study revealed an excellent reproducibility from one batch to another. Details are presented in Tables 102.4 and 102.5. Upon the batches D, E, G, and H, the Pearson correlation of MTT values of the repeated experiments was contained between 0.94 and 0.98, and the Pearson correlation of IL-1α values of the repeated experiments varied from 0.92 to 0.99.

The Pearson correlation was also calculated for the 50 compounds tested with both protocols. Concerning the direct topical application test, runs D and E were taken into account for the PVS chemicals. An MTT Pearson correlation of 0.96 was obtained for this protocol, and the value was 0.97 for the *in vitro* patch-test protocol. The corresponding measures of variation (mean standard deviation) are 5.2 and 2.7%, respectively. In parallel, the IL-1α Pearson correlation for the 50 chemicals was evaluated as 0.75 and 0.65, respectively. Moreover, the mean standard deviation for IL-1α release corresponds to 4.3% of the mean nonanoic acid-treated positive control for the direct topical application test. For the *in vitro* patch test, the mean standard deviation value for IL-1α release is 15.6%.

The predictive capacity of both protocols for the testing of the 50 tested chemicals obtained in our laboratory is shown in Table 102.6. For the *in vitro* patch test particularly, multiple endpoint analysis allows an improvement of accuracy and sensitivity, but a decrease in specificity (specificity = 63.6%, sensitivity = 82.1%, and accuracy = 74.0%), compared to the single endpoint approach (MTT reduction only) (specificity = 72.7%, sensitivity = 55.6%, and accuracy = 64.0%). For the direct topical application test, multiple endpoint analysis allows an improvement of specificity, but a decrease in accuracy and sensitivity (specificity = 40.9%, sensitivity

TABLE 102.4
Correlations Between MTT Values of Repeated Experiments

Pearson Correlation	D	E	G	H
D	1	0.98	0.94	0.97
E		1	0.97	0.98
G			1	0.98
H				1

TABLE 102.5
Correlations Between IL-1α Values of Repeated Experiments

Pearson Correlation	D	E	G	H
D	1	0.92	0.92	0.94
E		1	0.92	0.93
G			1	0.99
H				1

TABLE 102.6
Summary of the Results Applied to Both Protocols for the 50 Tested Chemicals

	Direct Topical Application Test	*In Vitro* Patch Test
% Specificity	40.9	63.6
% Sensitivity	89.3	82.1
% Accuracy	68.0	74.0

= 89.3%, and accuracy = 68.0%), compared to the single endpoint approach (MTT reduction only) (specificity = 50.0%, sensitivity = 82.2%, and accuracy = 68.0%).

102.4 DISCUSSION

A multiple endpoint analysis, including percentage of cell viability (MTT reduction), histology, and IL-1α release, has been elaborated in an attempt to ensure the relevance and improve the quality of the test results. Among the families of compounds described in Sections 102.3.2, and 102.3.3, the chemicals of the first family mimic the negative controls; however, the chemicals of the fifth react like the positive controls. The classification of these chemicals as nonirritant or irritant, respectively, is therefore unambiguous since all three endpoints lead to the same conclusion. If all compounds reacted similarly, one single endpoint would have been enough to classify chemicals. However, a multiple endpoint analysis is a reassuring method, which not only permits multiple information but also reveals its usefulness for the chemicals of families two, three, and four. The second comprises chemicals, which allow high epidermal cell viability, and a normal histology, but which provoke an increase in IL-1α release. According to our prediction model, chemicals belonging to this second family are classified as nonirritants. However, the increase in epidermal IL-1α release could be an early sign of skin irritation. These compounds may be irritant over a prolonged or repeated application, or on weakened epidermis.

The chemicals of the third family provoke tissue necrosis and a higher amount of released IL-1α compared to H_2O-treated negative control; however, cell viability remains higher than 50%. This class therefore contains irritants. MTT reduction is an efficient cell viability test when the whole tissue is necrosed. Then, no or little mitochondrial activity is observed leading to a strong decrease in the amount of formazan blue crystals in comparison to the negative controls. However, when suprabasal cell layers are necrosed while the basal cell layer remains viable, a normal MTT reduction takes place, resulting in a percentage of cell viability comparable to negative controls (Meloni et al., 2002). Moreover, some other false test results can be due to interactions between MTT and chemicals. This was the case for eugenol in the four repeated experiments on (living) cultures using the direct topical application protocol: the MTT values were contained between 66.5 and 75.9% of the H_2O-treated control, although histology showed necrosis, and the level of released IL-1α was high (≥143.9 pg/mL). This elevated MTT value was due to the interaction between eugenol and the MTT solution, since the application of eugenol on killed cultures induces an OD value, which is 82.8% compared to the viable untreated control tissue. On the contrary, we observed that the MTT values from eugenol-treated cultures in the patch-test protocol are contained between 12.0 and 17.5% of the H_2O-treated control. This could seem paradoxical, but it is necessary to remember that a smaller quantity of compound is applied in this protocol, and, moreover, that the patch partially absorbs the compound. In the case of eugenol, in patch testing, our hypothesis is that 4 h incubation is sufficient to allow complete necrosis of the tissues and increased IL-1α release, but it did not allow eugenol to reach the MTT solution and interact with it. On the contrary, potassium hydroxide 5% did not provoke any interaction with the MTT neither on living tissues nor on frozen-killed controls, although its direct contact with MTT solution provokes an OD value of 1.6. Concerning heptanal, the third and last compound which showed interaction with MTT, MTT values of 35% of the H_2O-treated control were observed during the experiments on living tissues. This is completely comparable to the results obtained on frozen-killed controls. Although it is cautious to proceed to additional controls such as MTT interaction with chemicals, multiple endpoint analysis also allows to detect false viability measurements. Moreover, the classification according to our prediction model is not modified, even when the interaction with MTT is significant. In particular, it allowed to classify eugenol as irritant (with the direct topical application test protocol) although it would have been impossible to conclude with a single MTT endpoint approach.

The fourth family includes chemicals that induced low cell viability and tissue necrosis, but that did not provoke any significant increase in the IL-1α release. Therefore, most of the compounds of this class are highly irritant or corrosive. The small amount of released IL-1α could be due to the fast and massive destruction of the tissues, which did not have time to release cytokines. Another explanation could be the direct destruction of the cytokines by the chemical. Most of these compounds belong to the strong acid/base family. The epidermal tissues are severely damaged, and necrosis is provoked by these compounds. They can thus penetrate more easily into the epidermal tissue and dissolve in the defined nutrient medium. This passive diffusion of the compound through the tissue to the medium was visually detected by the modification of the color of the medium.

Furthermore, some differences are observed between the test results of the two *in vitro* protocols described. First, MTT values are in most cases higher with the patch-test protocol. Accordingly, histology sections present a less severe necrosis. One possible reason for this is the reduced amount of chemical applied in the patch test in comparison with the direct topical test. A double quantity per square centimeter is applied on the tissues in this latter test. The quantity applied in the direct topical application test has been chosen to mimic published *in vitro* test protocols (Fentem et al., 2001), and for its capability to cover uniformly the whole surface of the epidermis, whatever the texture of the compound to be tested. In the *in vitro* patch test, the quantity of chemical applied was defined proportionally to the human patch test described by Basketter et al. (1997). Moreover, the structure of the Hill Top chamber itself is responsible for a partial absorption of some chemicals, reducing even more the amount of chemical that is in contact with the epidermis. It seems that the tissues necrosed more slowly (in the case of irritant chemicals) compared to the direct topical application test. False negatives may result from these two parameters in the patch-test

protocol. In particular, in the case of the compounds of the sixth class, the increased MTT values may represent overestimations since high percentages of cell viability are observed although the histology sections show necrosis.

However, although the chemical's effects seem to be less severe with the patch-test protocol, the application of an empty patch alone is responsible for a four- to fivefold increase in the basal level of IL-1α release. This increase is probably due to the ratio tissue surface/medium volume that was double for 0.63 cm² tissues compared to that for the 4 cm² tissues. Also, the topical application of an empty patch as well as some occlusive effects due to the patch induces a slight increase in basal IL-1α secretion.

The principal interest of *in vitro* experiments is not only to obtain reproducible data using more convenient, and more ethical test protocols, but also to produce useful indications on the human skin irritation potential of raw materials and finished products. The result of the comparison between *in vitro* and *in vivo* data is heterogeneous (Tables 102.2 and 102.3). Several chemicals were classified differently. On one hand, there are the compounds for which all *in vitro* and *in vivo* data corroborate, and on the other hand, the chemicals for which *in vitro* classifications conflict with those obtained *in vivo*; or even more, chemicals for which *in vivo* rabbit data are in opposition to human *in vivo* data. Concerning the PVS chemicals, we observed that our results resembled those performed with other epidermal models in most cases. Notably, dimethyl disulfide, *cis*-cyclooctene, 2-methyl-4-phenyl-2-butanol, and 2,4-xylidine were classified as irritant *in vitro* and nonirritant by the rabbit test. Lilestralis has been classified as nonirritant with our two *in vitro* test protocols, although the EU and the OECD data filed it as an irritant. However, the documentation available from its suppliers does not mention any irritant properties for this compound, but only sensitizing effects. All results shown here are relative to the sample of chemicals obtained. Therefore, the comparisons made with *in vivo* and *in vitro* test results are only indicative, as other batches of chemicals were tested. Moreover, methyl palmitate, which was tested on both rabbits (Fentem et al., 2001) and humans (Basketter et al., 1997), is classified as irritant and nonirritant, respectively. All *in vitro* tests, like the *in vivo* patch test, classified this compound as nonirritant. Interspecies differences could be the explanation.

In vitro reconstructed human epidermal tissues mimic the biophysical properties of *in vivo* human epidermis. However, the reconstructed epidermis seems more sensitive to some families of compounds. Although 17 days' air-lifted tissue cultures feature a fully differentiated stratum corneum (Rosdy and Clauss, 1990; Fartasch and Rosdy, 1996), and a normal lipid composition (Ramdin et al., 2001), their barrier function seems to be less efficient (Gysler et al., 1999; Garcia et al., 2000) compared to adult skin samples, leading to a higher sensitivity to chemicals. This higher relative permeability may correspond to the epidermis of a newly (17 days) reepidermised wound. This increased sensibility is considered as an advantage by Jones et al. (2003) and Garcia et al. (2000). Thus, when no toxicity is observed for a compound tested on reconstituted epidermis *in vitro*, the toxicologist can be confident about its safety regarding human use. Reconstituted epidermis could therefore be used according to the principle of precaution. Furthermore, *in vitro* tests using reconstructed epidermis present reproducible results leading to an unambiguous classification for almost all the tested chemicals.

Although the identification of the chemical's potential hazard is of the highest importance for industries and consumers, its classification is difficult. Human data are certainly the most informative, but they are available for only few chemicals, and this depends on the protocol used, the age (Robinson, 2002), the anatomical site (Lee and Maibach, 1995), and the seasonal variability (Basketter et al., 1996; Robinson et al., 1998; Geier et al., 2003). On the contrary, animal data are more easily available, but the protocol used, the organism, and even the laboratory may provide different results as shown by Weil and Scala (1971). Today, not only the relevance of the animal tests to assess human potential hazard is discussed but also the lack of reproducibility makes them even more questionable.

We cannot assure that our current protocols using 3D-reconstituted human epidermis are perfect to predict human skin irritation, but in addition to the classical advantages of *in vitro* methods, such as great convenience and reduced costs, reproducibility is strongly increased compared to other methods. This reproducibility is seen not only for a given product on repeated experiments but also by individual endpoint measured for each tested compound. In our experiments using the PVS chemicals with the direct topical application protocol, the Pearson correlation is contained between 0.94 and 0.98. The MTT values show that they are almost always of the same magnitude, not just under or over 50% of viability. In the same way, we could make very similar histological observations for a chosen chemical. The statistical comparison of the IL-1α results shows that even if the amount of released IL-1α is not always of the same range for the irritant compounds (Pearson correlation for 50 compounds of 0.75 for the direct topical application test, and of 0.65 for the *in vitro* patch test), chemicals can be classified into two classes. One contains the compounds that always present an amount comparable to that of the negative control, and the other that exhibits a significant increase in the amount of IL-1α compared to the negative control. Such a reproducibility has never been shown with the Draize test, nor with the human patch test. Because of this strong reproducibility, the human *in vitro* epidermis already represents the tool of choice for screening compounds for their skin irritation potential.

Interestingly, note that the results obtained in our laboratory with the *in vitro* patch-test protocol met the specificity, sensitivity, and overall accuracy performance criteria ($>60\%$) defined for the ECVAM prevalidation study described by Fentem et al. (2001) (Table 102.6). Moreover, a recent study performed by Kandárová et al. (2004) revealed that dimethyl disulfide had been improperly tested *in vivo*. Consequently, the real classification of this compound is unknown.

In parallel, methyl palmitate presents an ambiguous *in vivo* classification according to the literature (see Table 102.1). If we remove these two chemicals, specificity obtained with the *in vitro* patch test increases to 71.4%, sensitivity to 85.2%, and accuracy to 79.2%. Consequently, our *in vitro* patch test should be accepted for formal prevalidation by ECVAM.

However, all these test results are relative to the samples and lot numbers of the tested compound. We observed important variability in test results when certain compounds (lilestralis, hydroxycitronellal) came from different suppliers (unpublished data). For official validation studies, great care should be taken to control the quality of the reference compounds tested. Transferability being one of the parameters for ECVAM validation, it is indisputable that our encouraging intralaboratory results should be followed by an interlaboratory study. On the contrary, performance of the direct topical application protocol was disappointing compared to the *in vitro* patch-test protocol, and other published data. Taken together, this dataset provides a platform for further mechanistic and validation studies. We do not wish to overgeneralize these data; judgment will continue to be required when extrapolating such information for new chemicals in terms of their complex uses in biology. Moreover, SkinEthic epidermis is also involved in the current ECVAM skin irritation validation studies using the 15 min direct application time followed by a washing step and a 42 h incubation (Kandárová et al., 2006).

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103 Identifying Source of Textile-Dye Allergic Contact Dermatitis: Guidelines

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When a dermatologist concludes that the cause of a patient's allergic contact dermatitis (ACD) is a fabric dye, two major challenges follow: the first is to assist the patient in identifying the offending textile product(s); the second is to provide directions so that he/she can avoid the purchase of skin contact textile products that will cause a recurrence of his/her ACD skin lesions. Explaining how to identify the offending product(s) and purchase textile products that will not cause a recurrence of skin lesions can be daunting because textile products are rarely labeled to reveal their colorants. The only fabric "ingredient" revealed on textile labels is fiber content, information required by many countries including the United States, European Union countries, Japan, and South Korea.

Because many dermatologists are unsure about what directions to provide for their patients and the literature has revealed no written procedures, our objective is to provide a guideline for identifying which textile products/fabrics owned by patch-test-positive patients are the most likely to contain the dyes to which each is patch-test positive. We present this procedure in such a manner that patients can also use it to avoid the purchase of skin contact textile products most likely to cause a recurrence of their ACD skin lesions. The procedure involves four sequential steps.

103.1 IDENTIFY ALL COLORED TEXTILE PRODUCTS THAT CONTACTED THE AFFECTED SKIN AREA

The first step is for the patient to collect or list all colored fabric textile products that contact the skin area where his/her skin lesions appear. It is important not to eliminate potential ACD allergen-containing items during this first step. Patients need to think about the various locations where garments are kept in order to consider all items that touch their skin where lesions appear.

To assist in the listing or rounding-up task, we include Table 103.1 that provides a checklist of categories of textile products that contact various skin areas. During this process each patient should (a) consider all garments, bed linens (sheets, pillowcases, comforters, blankets), and other household textile products that contact their skin (towels, carpets and rugs, and upholstered furniture and car seats), (b) include skin contact products made from solid-colored fabrics including black (which some would argue are not colored fabrics) and those made from multicolored fabrics, (c) include long-time use or frequent-use items because these are as likely or even more so to contain the ACD allergen as "newly acquired" items due to the length of time that dye allergens

Hatch, K.L., Motschi, H., and Maibach, H.I., Identifying the source of textile dye allergic contact dermatitis—a guideline, in *Exogenous Dermatology*, 2(5), 240–245, 2003. [Karger]

TABLE 103.1
Product Categories to Consider Based on Location of Lesions

Upper Torso	Lower Torso
Back/Chest/Axillae	Stomach/Buttocks
Dresses	Pants/trousers
Shirts/Blouses	Dresses
T-shirts	Thermal underwear
Undershirts	Slips
Slips	Underpants/drawers
Shapewear	Warm-up suit bottoms
Warm-up suit tops	Pajamas
Pajamas	Legs
Thermal underwear	Socks
Sweaters	Hosiery
Swimwear	Dresses
Athletic wear	Skirts
Arms	Pants/trousers
Garments listed above with sleeves	Feet
Neckline	Socks
Garments listed above with collars	Hosiery
Jackets/coats with collars	Sock liners
Scarves (decorative and functional)	Shoes with fabric lining
Head	
Hats	
Hat bands	
Sheets	
Pillowcases	
Blankets	
Decorative pillows	

Note: No upholstery fabrics are included. There is no evidence to date that upholstery fabric is a source of an ACD allergen.

Source: Hatch, K.L., Motschi, H., and Maibach H.I., *Exogenous Dermatology*, 2(5), 240–245, 2003. With permission.

need to cause sensitization, and (d) include those outerwear garments that contact skin areas where lesions appear. An outerwear garment with a collar might be the cause of lesions in the neck area, one with sleeves longer than those of the garments worn underneath might cause lesions on the arms, wrists, or hands, and one with pockets might cause lesions on the hands.

103.2 NARROW THE NUMBER OF SUSPECT ITEMS USING FIBER COMPOSITION AND PATIENT PATCH-TEST RESULTS

The second step begins with finding the fiber composition of each item collected or listed. Fibers are the fundamental material unit in any fabric; they are the basic "ingredient." Information on fiber composition is most frequently located on a sewn-in label, often along with care information (laundering or dry-cleaning instructions) inside most garments, and along the hems of bed linens and towels. Garments that usually do not have information on fiber content attached to them are women's hosiery, as well as men's, women's, and children's socks. In these cases, the patient has to recall what the stated fiber composition was on the packaging at time of purchase.

The reason for finding information on fiber composition for each item is that it is the best information available from which one can reasonably deduce the colorant class or classes to which the colorant(s) on the fabric belong(s). As shown in Table 103.2 by the use of X in those locations where a fiber can be dyed with dyes named in the table header row, no fiber can be dyed with every type/class of dye. For example, cotton fibers can be colored with dyes in the direct, reactive, azoic, vat, sulfur, mordant, and pigment classes, but they cannot be colored with dyes in the basic and disperse classes and rarely with dyes in the acid class. Polyester fibers can be colored with dyes in the disperse and pigment classes but not with dyes in other classes. Further, as shown in Table 103.2 by the use of "X-mod," certain fibers can be modified to accept dyes in classes other than the "usual" classes. For example, polyester fiber when modified accepts basic dyes, and spandex, a fiber always combined with another one to make a fabric and usually dyed with disperse dyes, can be modified to accept dyes in the acid class and in the direct class.

Our recommendation is that each patient sort the textile items in his/her suspect pile or list by whether the item is made from a one-fiber content fabric, using two classes of fiber in the fabric, or made with two or more fabrics, each fabric in the item having a different fiber composition. Examples of items in the latter group would be lined garments such as coats, suit and outerwear jackets, tailored dresses, and slacks because the lining "inner fabric" usually has a different fiber composition than the "shell" or outer fabric.

Then, the single-fiber fabric group should be divided into a 100% cotton group, 100% acetate and triacetate fabric group, a 100% polyester group, a 100% nylon group, a 100% silk group, 100% wool group, and 100% olefin (polypropylene) group. There will be no 100% spandex group or 100% rubber group as these fibers comprise only a small proportion of the weight of any fabric. The multifiber fabric items should then be divided into (a) a polyester/cotton blend group, (b) those containing spandex, and (c) a miscellaneous group. No subdividing is needed at this time in the multifabric group.

Now *all dye-positive patients* can remove from their suspect group those items (a) whose fiber composition is 100% cotton, 100% flax (linen), 100% ramie, 100% rayon (viscose), and 100% lyocell, (b) made of fabrics with any combination of these fibers, (c) made of spandex and cotton fabrics, and (d) made of 100% polypropylene (Figure 103.1). The cellulosic-fiber items can be removed as suspects because they may only be colored with colorants from the direct, vat, azoic, sulfur, mordant, pigment, and reactive classes. Within the six first named colorant classes only three colorants, among the thousands of colorants in these classes, have been identified as ACD allergens [1,2]. By name those dyes are Direct Orange 34 (CI 40215), Direct Black 38 (CI 30235), and Vat Green 1 (CI 59825). In regard to the reactive dyes, many have been identified as ACD allergens [1,2]. However, once applied

TABLE 103.2
Pairing Colorant Class to Fiber Classes

Fiber Groups Based on Origin and Polymer Chemistry	Class Names	Acid	Basic	Disperse	Direct	Reactive	Azoic/Vat/ Sulfur	Mordant	Pigment
Fibers composed of cellulose polymers	Cotton, Coton/Baumwolle	Some[a]			X	X	X	X	X
	Flax (linen)				X	X	X		
	Ramie					X			
	Rayon/Viscose				X	X	X		X
	Lyocell				X	X	X		X
Fibers manufactured with chemically modified cellulose	Acetate/cellulose acetate			X					X
	Triacetate			X					
Fibers composed of protein or polyamide polymers	Wool	X			X	X		X	X
	Cashmere	X			X				
	Mohair	X							
	Angora	X							
	Camel's hair								
	Silk/soie	X			X	X			X
	Nylon/polyamide	X	X-mod	X-mod	X	X			X
Fibers composed of synthesized polymers (the manufactured synthetic fibers)	Polyester		X-mod	X					X
	Acrylic/polyacrylonitrile	X-mod	X						X
	Olefin/polypropylene			X					
The manufactured synthetic stretch fibers	Spandex/elastane	X-mod		X	X-mod				X
	Rubber[b]								

Table information compiled from Refs. 4–6.

Note: The origin of fibers is either from nature or from manufacturing. Natural fibers are produced by plants and animals. Manufactured fibers are derived by a process of manufacturing from any substance, which at any point in the manufacturing process, is not a fiber. Manufactured fibers made from synthesized polymers form the manufactured synthetic class. The fiber class names shown include those required in the United States and other countries. A manufacturer may elect to use several class names for the same fiber so the product will meet the labeling standards of several countries. Mod. = Modified form of the fiber that can be colored with this dye.

[a] A number of acid dyes will color cellulosic fibers but acid dye is not usually the dye of choice. Even in the dyeing of combination fabrics of silk, wool, or nylon with cotton, the cotton is dyed with direct dye and the other fibers with acid dye.

[b] Rubber fiber may also be manufactured from natural rubber that is obtained from rubber trees.

to fabric they cannot be the cause of ACD because they are covalently bonded to the cellulose polymers, and any excess dye on a fabric has a valence state that destroys its ability to be an ACD allergen. The cellulosic-fiber and spandex combination fabrics can be removed from the suspect list because the spandex in these fabrics is not dyed. Polypropylene items can be removed as suspects because when colored the disperse dyes are added to the fiber spinning solution trapping the dyes within the fiber. In other words, there is no disperse dye available for transfer to the skin.

As shown in Figure 103.1, patients who are patch-test positive to *disperse dyes only* can remove items having the following fiber compositions from their suspect list: 100% wools (including cashmere, mohair, angora, camel's hair, and alpaca), 100% silk, and 100% acrylic. The fabrics made of wool (including the specialty wool fibers), silk, acrylic, and combinations of these fibers are removed as suspects because they are not dyed with disperse dyes (Table 103.2). Items containing polyester fiber with spandex may be eliminated as suspects even though they are dyed with disperse dyes because such fabrics are given a "reductive clear": a process that removes disperse dye on the fiber surfaces, leaving all disperse dye molecules embedded within the fibers, thus not available for transfer to the skin. Items that disperse-dye-positive patients must keep on their suspect list are those made entirely or partially from (a) polyester fibers/fabrics, (b) acetate (cellulose acetate) fibers/fabrics, (c) triacetate fibers/fabrics, (d) nylon fibers/fabrics, and (e) combinations of these fibers.

Disperse-dye-positive patients are likely to have a considerable number of suspect items because polyester fiber is a component in many fabrics: business and casual dresses, slacks, blouses; T-shirts; washable uniforms for nurses, waitresses, and mail and package deliverers; fleece jackets (Polartec® for example), and bed linens (sheets, pillowcases, coverlets). Acetate (cellulose acetate) is a popular lining fabric in lined jackets and slacks and in fancy/formal dresses. Triacetate and nylon is a fiber combination often in fabrics for women's intimate apparel and sleepwear.

As shown in Figure 103.1, patients who are patch-test positive to *acid dyes only* can remove the items having the following fiber contents from their suspect list: (a) 100% polyester, (b) 100% acetate, (c) 100% triacetate, (d) combinations of these fibers, and (e) polyester blends such as

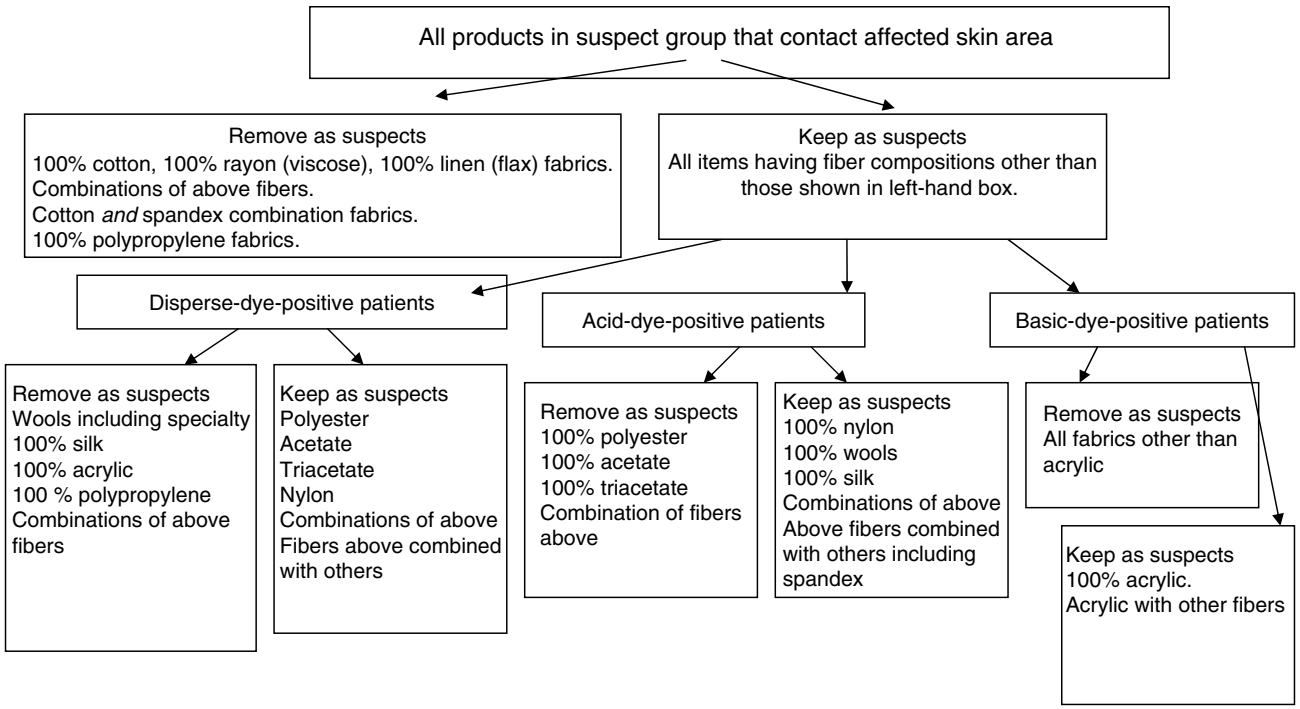


FIGURE 103.1 Guideline for determining which items are the most likely to be the source of a patient's ACD allergens. (Hatch, K.L., Motschi, H., and Maibach H.I., *Exogenous Dermatology*, 2(5), 240–245, 2003. With permission.)

polyester/cotton, polyester/rayon, or polyester/wool. These fabrics will not contain acid dyes (Table 103.2). Items that must be kept on the suspect list are those made partially or entirely from (a) wool (sheep wool), (b) specialty wools (angora, cashmere, etc.), (c) silk, (d) nylon, and (e) combinations of these fibers because these are fabrics that are most likely to contain acid dyes. Wool/spandex, silk/spandex, and nylon/spandex fabrics will contain acid dyes because the spandex in these fabrics will be the type modified to accept acid dyes.

Items likely to be included in the suspect list of the acid dye patch-test-positive patients are (a) sweaters, business suits, tailored dresses, and dry-cleanable uniforms (military, band, airline pilot) because such items are often made from wool and specialty wool fabrics; (b) expensive blouses, dresses, suits, scarves, women's underwear and nightwear, adult-sized thermal underwear, and sheets as these are often made using silk fabrics; and (c) women's sheer hosiery, men's socks, and windbreaker jackets as these items are often made from nylon fabrics.

Patients who are patch-test positive to *basic dyes only* can remove most items from their suspect list because basic dyes are only used to dye 100% acrylic fabric and fabrics containing acrylic fiber along with either polyester or nylon fiber. As shown in Table 103.2, polyester fiber and nylon fiber are modified to accept the basic dyes. These modified fibers are combined with acrylic to make the blended fiber fabrics. Items most likely to remain on the suspect list will be blankets, throws, sweaters, socks (especially sports socks), thermal underwear, mittens, gloves, winter-type scarves, and sweat suits.

Not shown in Figure 103.1 are the "keep" and "remove" lists for patients with positive patch tests to dyes of more than one type. These patients can determine their listing by combining the "keep as suspect" list in Figure 103.1. For example, patients who are patch-test positive to dyes in the disperse and acid dye classes will combine the items shown in "keep as suspects" under the disperse-dye patient heading and those in the "keep as suspects" list under the acid-dye-positive patient heading.

103.3 CONSIDER THE DYE FASTNESS OF EACH SUSPECT ITEM

The third step is to consider the dye fastness of each item in the suspect group. Dye fastness is a measure of the ability of the fabric to retain dye molecules under various conditions—when the fabric is rubbed (called wet crock fastness when the fabric is moist and called dry crock fastness when the fabric is dry), when the fabric is saturated with perspiration (called perspiration fastness), and when saturated with water (called wet fastness or wash fastness). Generally, fabrics with poor dye fastness when purchased are more likely to be a culprit than fabrics with good dye fastness at the time of purchase. The phrase "at time of purchase" has been used because dye fastness changes (usually improves) as a textile product is used, because dye molecules may be lost during laundering, as the fabric is abraded (rubbed), and as dye molecules are destroyed by the sun, ozone, and other agents. Therefore, over time less dye is available for transfer to the skin. Considering dye fastness does not allow items to be removed from the suspect list but allows items to be grouped as "more likely suspects" and "less likely suspects."

103.3.1 Wet Fastness and Color Bleeding

A good place to start is to recall whether any fabric on the suspect list caused the laundry water to become colored, or caused other items in the same wash load in which they were included to be stained, whether one observed such a color "bleed" from one area of the fabric onto another. If there are such items, they should be placed high on the suspect list as there was/is dye available for skin transfer. Then, care labels on the items in the suspect list should be read. When the care label states "wash separately" or "wash with like colors" that indicates potential poor color fastness. Such items should also be placed high on the suspect list.

103.3.2 Fastness to Rubbing

Often we do not observe that colorant is being lost when a fabric is rubbed. Fastness to rubbing is important because fabrics are continuously rubbed against the skin when in use. A quick test to determine the availability of dye to transfer is to rub the suspected fabric with a white swatch of cotton fabric. Hold the white swatch over the index finger and place the colored fabric on a horizontal surface. Have someone hold the colored fabric, while you rub the surface with the white fabric. Bear down and move the index finger back and forth about 10 times. Note whether any dye has transferred to the white swatch (the white cloth is now colored). (This simulates what is known as a dry crock test.) Then wet the white fabric, place it over your index finger in another location of the white cloth and again rub the colored fabric. (This simulates the wet crock test.) Again assess whether dye has been transferred (whether the white fabric became colored). Fabrics with poor crock fastness should be placed in the "more likely suspect" group and those with good crock fastness in the "less likely suspect" group.

103.3.3 Fastness to Perspiration

In a laboratory, fastness to perspiration is done by placing the colored fabric in an artificial perspiration solution and then bringing this swatch into contact with a white cotton swatch. The assembly is placed in an oven. On completion, the white fabric is observed to determine whether there has been dye molecule transfer. This test is difficult to simulate but in its place, one can recall when an item in the suspect list caused an undergarment to be stained with color in the underarm area, the interior elbow, or at a waistline. These items should be placed in the "more likely" category.

103.4 EXAMINE THE COLOR (HUE, SHADE, INTENSITY) OF THE FABRIC

The fourth step in the process can be to sort the fabrics within the most and less likely groups by hue (color) so that the blue and black fabrics are at the top of the suspect list. The rationale for such a ranking is that black and blue fabrics often contain the blue and red dyes that are ACD allergens.

It is also possible to rank items on the suspect list by shade placing those items that are a dark shade of any hue (red, green, blue) higher on the suspect list than those of a light (pastel) shade. The rationale behind the ranking is that a higher concentration of dye molecules is required to achieve a dark shade of any hue and that the concentration of dye available for transfer is critical in determining whether sensitization and elicitation ever occur.

103.5 SUMMARY AND CONCLUSION

Identifying which textile items most likely contain the colorant to which the patient is patch-test positive is not an easy task, but one that can be accomplished by following the four major steps outlined in this article:

1. Identify all colored textile products that contacted the affected skin area
2. Narrow the number of suspect items using fiber composition and patient patch-test results
3. Consider the dye fastness of each suspect item
4. Examine the color (hue, shade, intensity) of the fabric

The only way to know which fabrics contain the culprit dye (the dye to which the patient is patch-test positive) is to send the most likely items to a laboratory for dye content analysis or patch test with a swatch from each of the suspected fabrics. Both methods result in rendering the garment/product unusable as the item will be cut to obtain fabric for the test. Nevertheless, this is encouraged. Dermatologists who suspect that a patient has a colored-fabric ACD are encouraged to contact any of the authors of this paper for possible submission of the suspect garment for dye content analysis. These data will be added to an ongoing study involving the identification of disperse dyes in the fabrics of patients who are patch-test positive to at least one disperse dye [3].

An additional step that might be taken after the fabric is known to contain a dye to which the patient was patch-test positive is to patch test the patient with a swatch of that fabric or with dyes extracted from that fabric. Our recommendation at this time is to wait until there are (a) a collection of garments that do contain the allergen to which patients are patch-test positive and (b) a written protocol to conduct the tests (patch test with fabric and with dye extracts). Then it should be possible to develop reliable procedures.

Patients may use the fabric fiber composition listings in Figure 103.1 in the appropriate "remove as suspect box" to assist in purchasing textile items. Additionally they may also look for the Oeko-tex label on garments. This label identifies garments that do not contain most of the known textile dye allergens.

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104 Functional Map and Age-Related Differences in Human Faces: Nonimmunologic Contact Urticaria Induced by Hexyl Nicotinate

Slaheddine Marrakchi and Howard I. Maibach

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104.1 INTRODUCTION

Age-related and regional variation studies of the human skin reactivity to various irritants have been reported [1–5]. Marked variation of the various areas of the face in reactivity to benzoic acid has been documented by Shriner and Maibach [6].

In the present study, hexyl nicotinate (HN), a more lipophilic compound than benzoic acid was utilized to induce nonimmunologic contact urticaria (NICU) in the same sites documented by Shriner and Maibach [6]. Blood flow changes were recorded to determine potential regional and age-related differences in cutaneous vascular reactivity to HN.

104.2 MATERIAL AND METHODS

104.2.1 Subjects

Two age groups were studied: 10 healthy volunteers in the young group, aged 29.8 ± 3.9 years, ranging from 24 to 34 years, and 10 in the older group, aged 73.6 ± 17.4 years, ranging from 66 to 83 years.

Exclusion criteria were a history of atopy and current antihistaminic drug use.

All the volunteers gave written consent and the study was approved by the local ethical committee.

Reprint from Marrakchi, S., and Maibach, H.I., Functional map and age-related differences in the human in the human face: nonimmunologic contact urticaria induced by hexyl nicotinate, *Contact Dermatitis*, 55(1), 15–19, 2006.

104.2.2 Methods

Eight regions (forehead, nose, cheek, nasolabial and perioral areas, chin, neck, and volar forearm) were studied in terms of stratum corneum turnover (dansyl chloride test) and pharmacodynamic response to HN.

Dansyl chloride 5% in petrolatum was applied for 16 h on the eight locations of the skin using 8 mm Finn Chamber aluminum discs (Epitest Ltd, Oy, Finland) [7].

After dansyl chloride patch removal, the subjects were allowed to acclimate to the examination room for 15 min, then baseline measurements were taken on the contralateral locations.

Baseline measurements of the cutaneous blood flow (LDF) were taken using a laser Doppler flowmeter (laser doppler flowmetry blood flow monitor, MBF3D, Moor Instruments, England) [8]. Blood flow measurements were not taken on the upper eyelid because of the potential effect of the laser beam on the retina. Blood flow was monitored at one measurement per second for 30 s and the values averaged.

Using a saturated absorbent filter paper disc (0.8 cm diameter) (Finn Chamber), HN 5 mM in ethanol was applied on the eight skin areas for 15 s to elicit NICU. Then blood flow measurements were taken every 10 min for 1 h to detect the maximum vascular response of the skin to HN.

Stratum corneum turnover was determined by detecting fluorescence on each skin site everyday using a UV lamp. The period for the fluorescence to disappear was considered as the stratum corneum turnover.

Room temperature and relative humidity were recorded each time a subject was studied. Room temperature during the young group study (20.3 ± 2.3°C) was significantly ($p = .042$) lower than during the older group study (22.1 ± 2.3°C).

Relative humidity during the young group study (52.6 ± 3.8) was significantly higher ($p = .009$) than during the older group study (46.5 ± 5.5).

104.2.3 Statistical Analysis

To compare the measurements of the various skin sites within each group, the analysis of variance (ANOVA) test for analysis of variance was used. The two-tailed Student's *t*-test for unpaired data was used to compare the differences between the two age groups.

104.3 RESULTS

104.3.1 Baseline to Peak Changes

Cutaneous reactivity to HN was assessed by the baseline to peak changes (peak = maximum LDF − baseline LDF). In some investigations, area under the curve was also considered to assess these changes [6,9,10], but as it was correlated to peak values [6], only the baseline to peak changes (peak) were considered in our study.

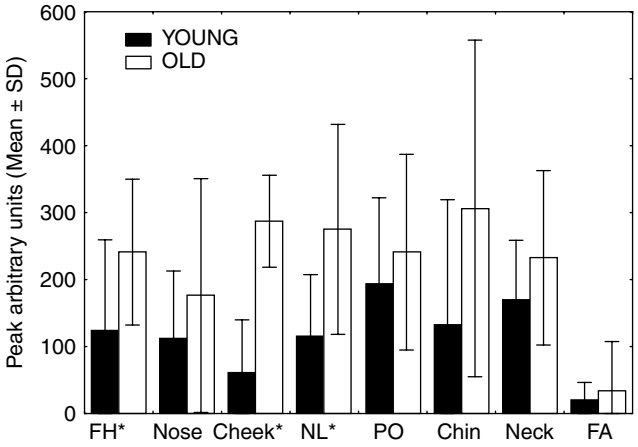


FIGURE 104.1 Baseline LDF to peak changes. Regional variation and age-related differences in the young and older groups. FH = forehead; NL = nasolabial area; PO = perioral area; and FA = forearm. *The regions where the difference between the two age groups was significant ($p < .05$).

104.3.1.1 Comparison Between Regions (Figure 104.1)

In the young group, the perioral area, followed by the neck, was the most sensitive to HN. The perioral and the nasolabial areas, the nose, forehead, and the neck were more sensitive than the forearm ($p < .05$). Perioral area ($p = .012$) and the neck ($p = .009$) were more sensitive than the cheek.

In the older group, all the areas of the face were more sensitive than the forearm. The chin followed by the cheek and the nasolabial area was the most sensitive. However, no difference in reactivity to HN was found between the various areas of the face.

The forearm was the less sensitive area in both groups.

104.3.1.2 Comparison Between the Two Age Groups (Figure 104.1)

Peak values were higher in the older group in three areas: forehead ($p = .047$), cheek ($p < .001$), and nasolabial area ($p = .012$).

104.3.2 Stratum Corneum Turnover

104.3.2.1 Comparison Between Regions (Figure 104.2)

The stratum corneum turnover was slower in the nasolabial area and the forearm in both the age groups.

The fastest stratum corneum turnover was shown in the perioral area and the chin in the young and in the chin and the forehead in the older group.

In the young group, nasolabial area and forearm stratum corneum turnover was significantly slower ($p < .05$) than forehead, cheek, perioral area, and the chin. The stratum corneum turnover was slower in the nose when compared to forehead ($p = .028$), perioral area ($p = .016$), and the chin ($p = .015$).

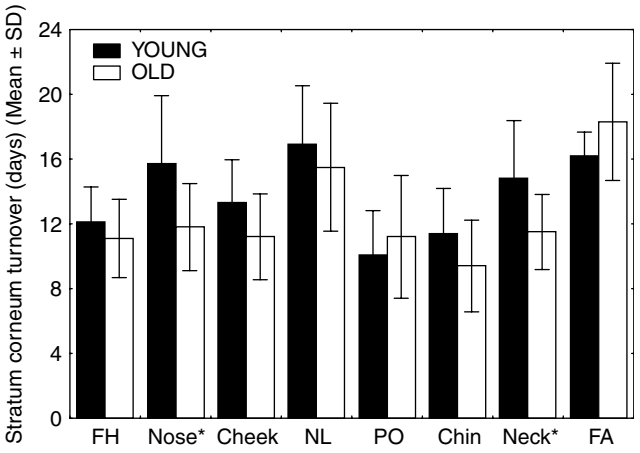


FIGURE 104.2 Stratum corneum turnover. Regional variation and age-related differences in the young and older groups. FH = forehead; NL = nasolabial area; PO = perioral area; and FA = forearm. *The regions where the difference between the two age groups was significant ($p < .05$).

The stratum corneum turnover was slower in the neck than the perioral area ($p = .004$) and the chin ($p = .029$).

In the older group, the forearm and the nasolabial area demonstrated a significantly slower stratum corneum turnover than the forehead ($p < .001$ and $p = .008$, respectively), the nose ($p < .001$ and $p = .025$), the cheek ($p < .001$ and $p = .011$), the perioral area ($p < .001$ and $p = .023$), the chin ($p < .001$ and $p = .001$), and the neck ($p < .001$ and $p = .013$).

104.3.2.2 Comparison Between the Two Age Groups (Figure 104.2)

In the nose and the neck, the stratum corneum turnover was significantly ($p < .05$) slower in the young than in the older group.

104.4 DISCUSSION

104.4.1 Vascular Response to HN (Peak)

In the young group, the highest vascular responses to HN were perioral area and the neck. In the older group, the chin, cheek, and nasolabial area showed the highest skin reactivity to HN.

This difference between the two age groups might be partly explained by the enlargement of the sebaceous glands in the elderly [11]. The UVA has been reported to induce sebaceous gland hyperplasia [12], which might lead to the enlargement of the sebaceous glands in the face when compared to other areas [13,14] and in the elderly when compared to the younger subjects [11,15].

Appendages may be an important factor in HN absorption, as the areas in the older group, where peak values were significantly higher than the young group, are known to have a high appendage density [16], and the enlargement of the sebaceous glands in the elderly [11] might explain that in the older group the absorption of HN seems to be higher where the appendage density increases.

Reviews and investigative studies have been published discussing the contribution of the various structures of the skin in the drug diffusion. Some note that the contribution of the appendages in the skin permeability to chemicals should not be overlooked especially during the early phase of absorption [17–19]. The appendageal route was reported to contribute to methyl nicotinate transport in the skin [5]. Using normal and artificially damaged skin (without follicles and sebaceous glands), Hueber et al. [20] demonstrated that the appendageal route accounts for the transport of hydrocortisone and testosterone, but is more important for this latter and more lipophilic compound. Illel et al. [21], studying rat skin, found that appendageal diffusion is a major pathway in the absorption of hydrocortisone, caffeine, niflumic acid, and *p*-aminobenzoic acid. Other studies [22,23] suggest that intercellular lipids composition is a major factor in barrier function.

However, one should keep in mind that skin reactivity to HN is probably not only the expression of the sole transcutaneous penetration of the molecule, but also the manifestation of individual variability in the vascular response to HN.

104.4.2 Stratum Corneum Turnover

The stratum corneum turnover was slower in the nasolabial area and the forearm in both the age groups.

Kawai et al. [24] reported in women a longer transit time in the volar forearm than in the face. In previous studies reviewed by Grove and Kligman [25], the stratum corneum turnover of the forearm was reported to decrease in the elderly. In our study the same trend was found although not statistically significant in all the areas, probably because of the shorter application time of dansyl chloride (16 h). Kawai et al. [24] did not find changes in the stratum corneum turnover in the face in the elderly.

So, in the volar forearm a photoprotected area, mainly the aging process is responsible for the differences between stratum corneum turnover between the age groups. The face is the most exposed area to the UV radiations. This could account for the reverse trend in the stratum corneum turnover although endogenous factors could also intervene. In the face, the stratum corneum transit time was shorter than in the protected area (the forearm) and in the older subjects who have received much UV radiation during their life than the young group. So it seems that the photoaging process probably decreases the stratum corneum transit time.

104.5 CONCLUSION

Many factors certainly account for the percutaneous absorption of the drugs. Besides the various physical parameters used in our study, noninvasive methods for the study of the appendageal density [26] and the stratum corneum lipids composition [27] should be considered to evaluate the influence of these two parameters on percutaneous absorption of chemicals.

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105 Is Contact Allergy Lifelong in Humans? An Overview of Patch-Test Follow-Ups

Ernest Lee and Howard I. Maibach

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Contact allergy in man is considered either to be lifelong or at least to last for years.[1] We examined follow-up studies on contact allergy, as evaluated by patch testing, attempting to quantify its natural history. In addition to utilizing Medline and the Science Citation Index (1960–2000), we concentrated our efforts on the following publications: *Contact Dermatitis*, *Dermatosen*, *American Journal of Contact Dermatitis*, *Archives of Dermatology*, *Journal of the American Academy of Dermatology*, and *American Journal of Clinical Dermatology.*

105.1 COLOPHONIUM

Farm[2] retested 83 patients after 9–13 years in whom contact allergy to colophonium had been diagnosed. Sixteen participants (19%) were negative to colophonium, and in seven the reaction was doubtful. We are uncertain of the biological significance of the doubtful reactions because test substances from another supplier were used. In addition, since colophonium is not particularly known to cause irritant patch-test reactions,[3] the doubtful reactions may in fact have represented contact allergy. The composition of this complex natural product may also have varied over the decade.

105.2 GOLD SODIUM THIOSULFATE

Bruze et al.[3,4] examined the development and course of test reactions to gold sodium thiosulfate. This patch-test response has a rate of approximately 10% when added to the standard series. Over 2 months, 10 patients were retested epicutaneously (e.c.) and intracutaneously (i.c.) with dilution series. All patients (100%) had a positive test reaction of some form during the retest. During the entire study, 26 positive e.c. reactions were diagnosed. Within the first week, 17 (65%) were recorded; in 10 days, another 9 reactions (35%) appeared. The patients with the latter reactions also had positive test reactions within the first week. After 2 months, 9 reactions remained. Out of 30 i.c. tests, 25 became positive within 1 week.

Although the authors did not provide information on the rapidity of the initial patch test to diagnose allergy, they did suggest that active sensitization can occur, leading to a more rapid response on retesting. To diagnose contact allergy to gold sodium thiosulfate, readings at 3 days might be supplemented by readings at 1 and 3 weeks.[4] All the patients remained positive for contact allergy to gold sodium thiosulfate from the point of their initial diagnosis to the time of this study.

105.3 NICKEL

Schubert et al.[5] restudied 104 nickel-positive patients 3 years later; 13 (12.5%) were then negative. Christensen[6] examined the prognosis in nickel allergy and hand eczema in two similar patch test studies performed 6 years apart. Fifty-two of 54 (96.2%) patients remained positive. Hindsen et al.[7] tested 30 women allergic to nickel on four occasions. No patient showed the same patch-test reactivity on all occasions, and the highest individual difference noticed was 250× for the four test occasions. Furthermore, two patients had negative test reactions on at least one test.

Modified from Lee, E., and Maibach, H.I., Is contact allergy in man lifelong? An overview of patch test follow-ups. *Contact Dermatitis*, 44, 137–139, 2001. With permission [Blackwell publishing].

105.4 COBALT

Rystedt[8] studied cobalt (Table 105.1). During a 5-year period, 286 of 4034 (7.1%) standard series–tested eczema patients reacted. Fifty (1.2%) showed isolated cobalt reactions (i.e., without simultaneous reaction to chromium or nickel). Three of the 50 patients (6%) who had originally shown isolated reactions to cobalt failed to react to cobalt on subsequent standard testing and were therefore excluded from the group to be tested with a serial dilution test (SDT). A follow-up study of 36 of these patients included an SDT: 15 of the 36 patients (42%) had negative reactions in the SDT.

105.5 DISCUSSION

In evaluating these results, other factors that may influence patch testing must be considered. There is individual variation in patch-test reactivity due to factors such as hormones, drugs, and ultraviolet radiation (UVB).[9–14,16,17] For example, there can be changes in trans-epidermal water loss (TEWL) and cutaneous blood flow during the menstrual cycle.[13,14] On the back and forearm, TEWL is significantly higher on the day of minimal estrogen/progesterone secretion as compared to the day of maximal estrogen secretion. There is also a higher baseline blood flow on the day of maximal progesterone secretion compared to the day of maximal estrogen secretion. One group found the skin response to challenge with sodium lauryl sulfate to be significantly stronger on day 1 than on days 9 through 11 in the menstrual cycle.[10] Since patch testing is at least a 4-day procedure, it is conceivable that more transient hormonal peaks than the aforementioned could also have an impact on its results. There are regional variations of patch-test response in nickel-sensitive patients.[15] Routine patch-test results can also be seasonally influenced through UVB.[16] Elicitation of positive patch tests may lead to an increased skin reactivity toward the same allergen when the patients are retested weeks later.[17] Studies were performed on the reproducibility of contact allergy, and a highly reproducible response was found; however, we are concerned about the low number of patients studied and the fact that there was only one retest.[18] As well, one longitudinal study of contact sensitivity in patients with atopic dermatitis found changed reactivity patterns.[19] In doubtful cases of contact allergy to metals, intradermal testing can be utilized.[20] At present, due to technical limitations noted here, we cannot state in quantitative terms whether contact allergy in man is lifelong or whether its patch test and clinical manifestations change. Taken together, in spite of a century of diagnostic patch testing, we are unable to state with reasonable certainty the length of delayed hypersensitized immunologic reactivity.

105.6 CRITERIA FOR FUTURE STUDIES

The following observations should provide data that might aid in the interpretation of future studies:

- Simple retest at the first positive to rule out excited skin syndrome
- Serial dilutions at both time periods to provide more quantitative data
- Utilization of the same test site (i.e., upper versus lower back), patch chamber, patch application, tape, and pure rather than complex allergen (i.e., nickel versus *Myroxylon pereirae* resin)
- Considering provocative use test (PUT)/repeated open application test (ROAT) to provide additional clinically relevant data
- Observations on the clinical course

In principle, serial dilutions and PUT are probably the most valuable in assessing clinical relevance. Serial dilutions provide an estimate of relative potency; the lower the concentration a subject reacts to, the less likely the result will be false positive due to irritation.

TABLE 105.1
Summary of Follow-Up Studies Involving Contact Allergy Remission of Allergy as Evaluated by Patch Testing

Allergen	Colophonium	Nickel		Cobalt
Frequency of positive patch-test reactions to allergen among patients	3–7%	6–7%	6–7%	7.1%
Duration	9–13 years	3 years	6 years later	5 years
Number of patients in follow-up study	83	104	63	36
Number of patients showing loss of reactivity	16 (19%) negative, 7 doubtful patch-test results	13 (12.5%) negative, 68 free from nickel dermatitis, 16 with a very mild eczema or dyshydrosis	30% were healed, but patch testing revealed a negative patch test in only 2 of 54 (3.8%) patients previously positive; patients with pompholyx-type eczema had worst prognosis; atopy made prognosis worse	15 (42%) negative reactions in the SDT; however, 11 of those demonstrated weak reactions in the previous standard test
Reference	2	5	6	8

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106 Irritants in Combination with Synergistic or Additive Effect on Skin Response: Overview of Tandem Irritation Studies

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106.1 INTRODUCTION

Occupational skin disease is an important health burden in society and causes a significant impairment in the quality of life of employees;[27] where the majority of the reported cases suffer from irritant contact dermatitis of the hands.[3] ICD is a nonimmunological local inflammatory reaction characterized by erythema, edema, or corrosion following single or repeated application of a chemical substance to an identical cutaneous site.[31] Studies have investigated and quantified the effects of single irritant exposures, in particular sodium lauryl sulfate (SLS), which has been studied as a model irritant.[4,6,13,15,17,33,38,39] However, irritation dermatitis in the workplace generally occurs with a combination of exposures to multiple irritants rather than with just a single irritant. Studies on ICD with multiple irritants have recently been studied to simulate conditions in the workplace and in consumers in a calculated and valid methodology. Various experimental designs have been used by research groups to analyze the interaction between multiple irritants in ICD, making it difficult to interpret and compare the data across studies in order to draw a conclusion.[2,4,6,9,13–15,17,28,33,38,39,41,45]

Recent studies have shown through "tandem" or sequential applications of different agents that the cutaneous response in ICD can be different compared to repeated exposures to a single irritant. The coined term tandem repeated irritation test (TRIT) has been reported as having potential in the study of repeated exposure to multiple irritants in contact dermatitis.[46] The results observed in TRIT studies have shown various modification of the cutaneous response compared to the response seen with exposure to single irritants. Depending on the pairs of irritants investigated, several types of cumulative irritation may result: (i) additive, a tandem response that equals the sum of the expected responses from each irritant alone; (ii) synergistic, a tandem response that is far greater than the sum of the expected response from each irritant alone; (iii) quenching, a tandem response much less than the sum of the expected response to each irritant alone. We review the development of tandem irritation studies and the possible mechanisms that may lead to the responses seen with such irritation studies. Knowledge of the trend in tandem irritation response can guide us in selecting subsequent pairs of irritants to use in future studies to elucidate the mechanism of action behind skin permeability barrier disruption in ICD.

106.2 MEASUREMENTS

Researchers have followed disparate procedures when conducting tandem irritation studies in the past, thus making comparisons across studies difficult. The most recent tandem irritation studies, however, have been performed using similar study designs.[14,28,41,45] Studies were performed in single-blinded, randomized manner, under standard laboratory conditions at room temperature 20–22°C and 30–40% relative humidity. Subjects underwent acclimatization periods of 15–30 min. Application areas are on the paravertebral midback or volar forearm, with 12 mm Finn Chambers (Epitest, Helsinki) under occlusion. The first irritant was applied on the filter discs, and removed after 30 min. The area was carefully rinsed with 10 mL tap water and blotted dry with paper tissue. After 3 h, the second irritant exposure was performed. This procedure was repeated for 4 days in each case at the same time of the day ±1 h.

Assessment of the irritant potential of chemicals in irritant contact dermatitis has been investigated through various methods: visual scoring, transepidermal water loss (TEWL), laser Doppler flowmetry, electrical capacitance, and skin color reflectance. Visual scoring has been performed in different ways. Several studies utilized the scale of Frosch and Kligman,[19] based on three main types of skin lesions: erythema, scaling, and fissuring (erythema: 1+ slight redness (spotty or diffuse), 2+ moderate and uniform redness, 3+ intense redness, 4+ fiery redness; scaling: 1+ fine, 2+ moderate, 3+ severe with large flakes; fissures: 1+ fine cracks, 2+ single or multiple broader fissures, 3+ wide cracks with hemorrhage or weeping). Whenever the visual score developed to a value ≥5 in a single test field (cutoff criterion), the exposure to the irritant was prematurely discontinued. Other studies employed the scale of Willis et al.,[48] using the erythema scale alone as their visual scoring criteria, with similar cutoff criterion.

TEWL, expressed in g m^{-2} h^{-1}, is an indicator of the water vapor pressure between the boundary layer of the skin surface and the ambient air. It reflects the integrity of the epidermal permeability function under normal or barrier-perturbed conditions such as in compromised or diseased skin. An evaporation meter is used in most of the recent tandem studies to measure TEWL in accordance with the Guidelines of the Standardization Group of the European Society of Contact Dermatitis (ESCD).[35] TEWL has been reported as the most reliable measurement in assessing skin barrier impairment compared to other bioengineering methods.[1] Gender, race, and age does not have a cause a significant difference on TEWL, with skin of forearm and back having similar results for TEWL. However, interrater and intrarater difference may exist between opposite arms, and before and after exposure.[35]

Laser Doppler flowmeter measures cutaneous blood flow. The Doppler-shifted light is backscattered from moving red blood cells, while unshifted laser light is backscattered from stationary tissues. The blood flow signal was expressed in relative and dimensionless values.[32]

Electrical capacitance indicates the hydration level of the skin and can be measured using a corneometer CM 825.[7]

Measurements of skin color were conducted using a chromameter with the guidelines from Elsner[11] or other published guidelines.[20,34] The instrument measures color reflectance and computes the chromatic dimensions of color by means of the L*a*b 3-D dimensional colorimetric system. To quantify erythema, the a* value is of specific interest in measuring the red (positive value) and green (negative value) distinction.

106.3 MECHANICAL IRRITANTS

Conditions that may support the induction of barrier disruption and subsequent ICD include mechanical and chemical irritation. We can distinguish the tandem irritation studies between mechanical and chemical irritation. Mechanical irritants in the workplace have been reported in the form of influences from air conditioning, climatic changes, temperature changes, airflow.[40,44] Chemical irritants, however, come in a vast array from detergents and perfume exposures in housewives to that of fruit acids in bakers.[3,39,42]

106.3.1 Temperature

Interactions between mechanical and chemical irritants have been studied since 1977 when Rothenborg et al.[39] described temperature-dependent irritant dermatitis from lemon perfume. In a study of an outbreak in cleaning personnel from using a lemon-scented detergent, it was shown that exposure to 43°C of the lemon perfume component citral proved to be a primary irritant (toxic) dermatitis even at 48 h after the exposure. This was in contrast to the reversible skin reactions seen with citral at 23°C which had reduced by 3 h after the exposure. A similar experiment to observe effects of low temperature was done by Halkier-Sørensen et al.[25] Findings reported a reduction in erythema, itching, but no apparent effects on local edema (Tables 106.1 and 106.2).

These findings led to subsequent experiments to assess temperature-dependent skin damage. Berardesca et al.[4] reported the effect of increasing temperature on surfactant (5% SLS)-induced irritation on the skin. Indeed damage was progressive according to increasing temperature. A supporting finding by Øhlenschlæger showed that by immersions of the forearm into 40°C 0.5% SLS, significant TEWL occurs as compared to at 20°C SLS. However, SLS-only immersion experiments showed a reduction in skin capacitance regardless of temperature at 40 or 20°C, even though TEWL did not show significant changes.[33] A subsequent study proposed that the anionic character of the detergent and temperature has a positive correlation to the irritant potential.[6] The study compared irritant effects by SLS with a milder detergent where 50% of the anionic molecules were replaced by nonionic molecules (lauryl glucoside). The solutions were then tested at 37 and 40°C, and compared to each other; the increase in temperature and anionic detergent content indeed caused a stronger response.

In contrast to the many studies conducted on the effect of an increase in temperature, few studies were reported to

TABLE 106.1
Mechanical Irritation Studies Simultaneously and in Tandem

Design	Irritant/Vehicle	Materials and Methods	Result	Reference
Simultaneous heat + irritant	Citral 10% in alcohol	Ventral forearm with Finn chambers. 20 min under occlusion. Evaluated by biopsy.	Strong toxic acute dermatitis only seen at high temperature (43°C).	39
Simultaneous heat + irritant	SLS in water	*In vitro* skin. 2 h immersion then at 75–90°C in oven by DSC. Evaluated by DSC, TEWL, and XRD.	Increased temperature causes disorganized lipid pattern on XRD. SLS decreases lipid *T*m.	38
Simultaneous heat + irritant	SLS in water	Ventral forearm. Open application then air dried. Evaluated by TEWL, erythema, colorimetry, and capacitance.	Increased TEWL, erythema colorimetry, and decreased capacitance seen at 20, 40, but not at 4°C.	4
Simultaneous heat + irritant	SLS in water; water	Arm immersion at 20 and 40°C. Evaluated by TEWL, capacitance, and erythema.	SLS reduces capacitance at 20 and 40°C. Increased TEWL at 40°C, not at 20°C.	33
Simultaneous heat + irritant	SLS (anionic) in water; SLS (anionic + nonionic) in water	Arm immersion at 37 and 40°C. Evaluated by TEWL, capacitance, and erythema.	More increase in TEWL, erythema and less of a decrease in capacitance at 40 than 37°C.	6
Simultaneous cold + irritant	Histamine 0.3%	Ventral forearm scratch test at room temperature and after ice cube application. Evaluate for erythema, SBF, and wheal.	Cold reduces erythema by 50%, reduces itch, but does not reduce wheal formation.	25
Tandem cold + irritant	SLS in water	Paravertebral midback with Finn chambers at 4 and 15°C. Evaluated by VS, TEWL, and colorimetry.	SLS/cold and cold/SLS decreases TEWL. No changes in other measurements.	15
Tandem airflow + heat + irritant	SLS in water	Paravertebral back with Finn chambers. Evaluated by VS, TEWL, and colorimetry.	Additive effect in airflow/SLS and SLS/airflow.	17
Tandem mechanical irritation + irritant + occlusion	SLS in water	Ventral forearm with Finn chambers. Evaluated by VS, TEWL, and colorimetry.	Occlusion (by water, glove, or SLS)/ mechanical irritation caused more increase in TEWL than mechanical irritation/ occlusion. Longer period of occlusion increases TEWL.	13

Note: SLS = sodium lauryl sulfate, DSC = differential scanning calorimetry, TEWL = transepidermal water loss, XRD = x-ray diffraction, VS = visual scoring, SBF = skin blood flow. Temperatures are reported in °C.

evaluate the effect of cold in relation to detergent irritation. A study on the relevance of low skin temperature in fish-processing employees showed that coldness induced on the skin by ice cubes to 15°C significantly reduce the symptoms seen with histamine scratch testing otherwise pronounced at room temperature.[25] A reduction was seen in itching, erythema, and skin blood flow. However, the extent of the wheal reaction stayed constant, meaning capillary permeability was not much affected by cooling. Fluhr[15] studied the effects of 0.5 and 1% SLS at 15 and 4°C, respectively, and its effects on TEWL, visual scoring, and skin color reflectance. Indeed coldness at both temperatures decreased TEWL and visual scoring; however, a decrease in redness by skin color reflectance was not observed.

This apparent protective effect of coldness on skin barrier impairment, however, is contradicted in another study by Halkier-Sørensen,[26] and a disruption in homeostasis is actually suggested as an influence of coldness.

However, the previously mentioned studies on the effects of temperature on skin barrier were not performed as tandem irritation studies, but rather in a combined fashion. Therefore, we could not evaluate for synergistic or additive interaction of temperature and SLS. Heated or cooled SLS was prepared at different temperatures and tested on the skin surface. Øhlenslæger et al. showed 0.5% SLS immersion of the forearm at 20 and 40°C, and then compared to water immersion at the same temperatures. A significant increase in TEWL was only observed as a combined effect of SLS and increased temperature and not when SLS or temperature increase was introduced alone. This is because increased temperature causes an increase in water flux through the stratum corneum (SC). Increase in water flux can increase penetration of SLS and subsequently increase the impairment of barrier function (Table 106.3).

106.3.2 Airflow

The last two studies published by Fluhr[17] on mechanical irritation involved testing the effects of airflow and temperature in tandem with SLS. Paravertebral back skin areas were exposed to airflow at different velocities at 24 and 43°C in combination with 0.5% SLS in a tandem repeat fashion. Temperature or airflow alone did not have a significant effect on TEWL in the tandem studies. It was also concluded that

TABLE 106.2
Chemical Irritation Studies Simultaneously and in Tandem

Design	Irritant/Vehicle	Materials and Methods	Result	Reference
Tandem chemical irritants	RA in ethanol. SLS in water	Ventral forearm with occlusive polypropylene chambers. Evaluated by TEWL, VS, SBF, and capacitance.	Synergistic effect in SLS/RA. Additive effect in RA/SLS.	9
Tandem chemical irritants	RA in ethanol. SLS in water	Paravertebral midback with Finn chambers. Tandem interval time was varied. Evaluated by VS, TEWL, and colorimetry.	Increased reaction at day 1–2, remains same at day 7, resolves at day 7–14 after the injury.	2
Tandem chemical irritants	SLS in water; toluene, undiluted	Forearm with Finn chambers. Evaluated by VS, TEWL, and colorimetry.	Synergy seen with SLS/toluene and toluene/SLS.	45
Tandem chemical irritants	SLS in water; *n*-propanol in water	Ventral forearm with Finn chambers. Evaluated by VS, TEWL, and colorimetry.	Limited additive effect seen with SLS/*n*-propanol.	28
Tandem chemical irritants	Ascorbic acid in water; acetic acid in water; NaOH in water; SLS in water	Paravertebral midback with Finn chambers. Evaluated by VS, TEWL, and colorimetry.	Additive effect in TEWL for ASC/SLS, ACA/SLS, and NaOH/SLS.	14
Tandem chemical irritants	Citric acid in water; lactic acid in water; malic acid in water; SLS in water	Paravertebral midback with Finn chambers, at pH 2 and pH 4. Evaluated by VS, TEWL, and colorimetry.	Quenching effect with lactic acid/SLS and citric acid/SLS at pH 2. Additive effects with other combination tandem studies.	41

Note: SLS = sodium lauryl sulfate, DSC = differential scanning calorimetry, TEWL = transepidermal water loss, XRD = x-ray diffraction, VS = visual scoring, SBF = skin blood flow, RA = retinoic acid, ASC = ascorbic acid, ACA = acetic acid. Temperatures are reported in °C.

both velocities (60 and 90 $m^3\ h^{-1}$) did not have a statistically significant effect on TEWL, although the authors noted that the slower airflow induced irritancy more rapidly. They concluded that exposure to airflow in tandem with SLS has an additive effect in skin barrier disruption (Figure 106.1).

106.3.3 Mechanical Irritation

A recent study investigated the effect of mechanical irritation with a brushing machine in tandem with water occlusion and occlusion with 0.5% SLS.[13] Irritation was then assessed by visual scoring, skin color reflectance, and TEWL. They concluded that mechanical irritation alone caused a mild, but significant increase of TEWL values on the last day of the experiment. Furthermore, occlusion followed by mechanical irritation induced a stronger barrier function impairment than mechanical irritation followed by occlusion. The study also showed that increase in length of occlusion time (by water, glove, or SLS) caused an increase in TEWL. This finding was attributed to an initial dissolution of SC integrity, especially by SLS.[24]

106.4 CHEMICAL IRRITANTS

Numerous studies on the effect of chemical irritants have used SLS as a model irritant in comparison to the more recently studied chemical irritants. A well-accepted dose-dependent response of SLS irritation has been reported. The mechanism behind disruption of skin barrier by detergents, especially SLS, has been thought to cause a decrease in lipid melting point and an increase in water diffusion.[38] Ribaud et al.[38] investigated the effects of SLS on the barrier function of *in vitro* skin tissue. SLS introduced lipid changes similar to heat treatments, which cause a decrease in lipid melting temperature from the critical micellar concentration of SLS (0.24%). A decrease in the intensity of diffraction pattern peaks with increasing SLS concentration was also observed. Both changes indicate a disorder pattern that develops in the intercellular lipid organization with the application of SLS on human skin. The mechanism of this permeability alteration remains *sub judice*. Barrier function of the SC is mainly due to ceramides; however, studies by Froebe et al. indicated that ceramides were not extracted by SLS.[18] When applied topically, SLS is an acute irritant, by a direct cytotoxic effect on keratinocytes, affecting both lipid and protein structures. SLS can induce damage of skin barrier function, exceeding 10 days after a single occlusive application.[12,29]

The subsequent tandem studies performed have used SLS as a model irritant for comparison. A study on the effect of retinoic acid (RA) on skin permeability by Effendy et al. quantified the irritant effects of 0.05% RA, 1% RA, and 1% SLS on normal skin in a 24 h assay, both alone and in tandem, that was monitored for 18 days.[9] Measurements were taken for visual scoring, TEWL, skin blood flow, and electrical capacitance. No statistically significant difference was found between results using 0.05 and 0.1% RA, suggesting a similar potency. SLS in tandem with RA produced a significant increase in TEWL compared to RA in tandem with SLS, or compared to repeated exposure of each irritant

TABLE 106.3
Additive, Synergistic, and Quenching Results of Tandem Studies

Interaction[a]	Tandem Irritants	Ratio of Experimental/ Calculated TEWL[b]
Additive	SLS/propanol	0.60
	SLS/ACA	0.69
	SLS/ASC	0.73
	SLS/NaOH	0.75
	SLS/Citric acid pH 4	0.59
	SLS/Lactic acid pH 4	0.55
	SLS/Malic acid pH 2 and pH 4	0.63
	RA/SLS	1.03[c]
Possible synergy	SLS/Toluene	2.86
	SLS/RA	1.54[c]
Possible quenching	SLS/Citric acid pH 2	0.48
	SLS/Lactic acid pH 2	0.50

[a] Interaction was arbitrarily defined by ratio of experimental/calculated TEWL values: (i) additive: $0.51 \leq$ ratio ≤ 1.50, (ii) synergistic: ratio ≥ 1.50, (iii) quenching: ratio ≤ 0.50. Day 5 TEWL values were used for ratio calculation, baseline values at day 0, when provided in the paper, were subtracted from the TEWL values.

[b] Calculated TEWL is obtained by taking the average of TEWL resulting from repeated tandem exposure to each of the irritants alone as TEWL from single exposure to each of the irritants were not provided in the papers. The ratio is then obtained by dividing the measured TEWL by the calculated TEWL.

[c] In case of RA, the calculated TEWL was obtained using the sum of TEWL resulting from single exposure to each of the irritants alone. The ratio is then obtained by dividing the measured TEWL by the calculated TEWL. Day 3 TEWL values were used for ratio calculation; baseline values at day 0, when provided in the paper, were subtracted from the TEWL values.

alone. However, exposures to single irritants were not performed twice with a 24 h interval over 2 days as the tandem experiments, but the single irritant controls were performed once over a 24 h period. RA exposures also increased the amount of scaling on the skin independent of SLS application. An increase in erythema, skin blood flow, and water loss (reduced capacitance) was statistically significant with SLS followed in tandem by RA compared to RA followed in tandem by SLS. They concluded that exposure to SLS may render the SC barrier highly permeable and allow a greater penetration of the following RA application. Interestingly, analyzing the data published by Effendy et al. by measuring the heights on graphs of TEWL, taking into account baseline measurements at day 0, SLS/RA tandem application caused an increase in TEWL by a factor of 1.8 of the sum of the TEWL by each irritant alone, whereas RA/SLS had an increase in TEWL by a factor of 1.03 compared to the sum on TEWL by each irritant alone. The interaction of SLS/RA in tandem seems to have a synergistic effect whereas the RA/SLS in tandem application shows an additive effect.

A following study was performed by Ale et al. on the tandem application of SLS/RA compared with SLS/ethanol, water/ethanol, and water/RA to confirm the synergism seen in the study by Effendy et al.[2] Measurements were taken for TEWL, capacitance, and skin color reflectance. The time intervals between the tandem applications were varied between 1, 24, 48, 96 h, and 1 and 2 weeks. The results showed a synergistic trend in SLS/RA in all variables, except capacitance, showing an antagonistic interaction for skin hydration. It was also noted that an increased reaction occurs at 1 and 2 days, remains at 7 days, and resolves 7–14 days after acute SLS injury.

The following tandem irritation study evaluated the effects of a tandem repeat study on 0.5% SLS and undiluted toluene.[45] The matched controls were performed with repeated exposures to a single irritant according to the tandem application

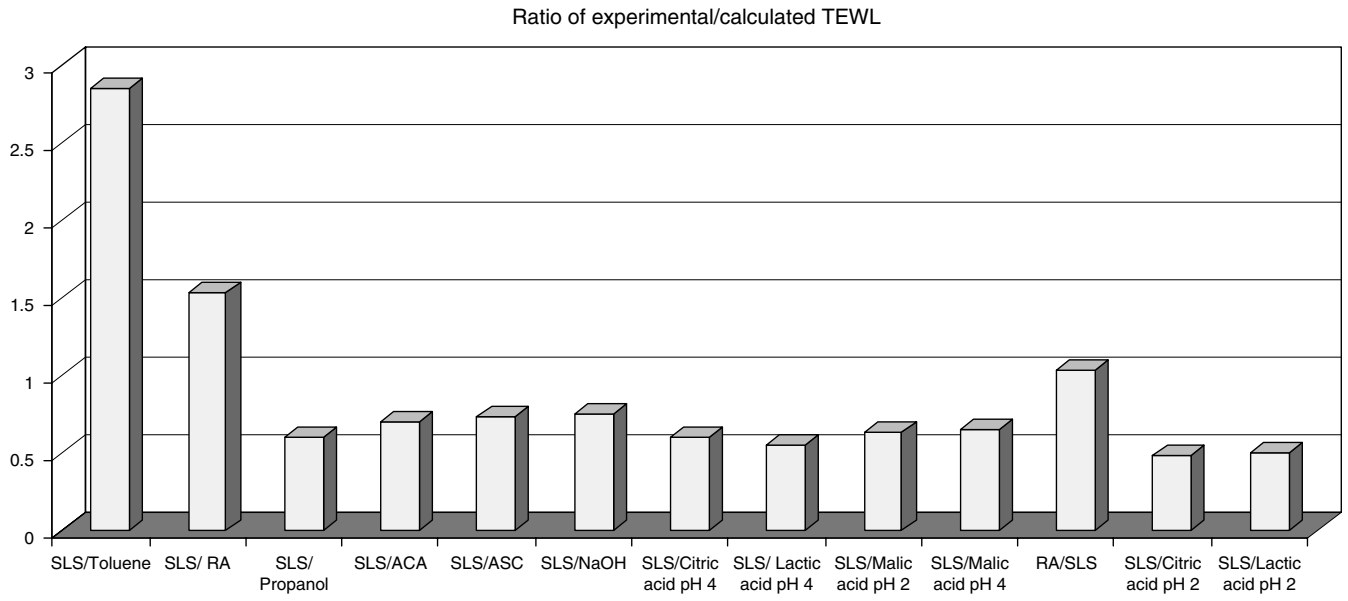


FIGURE 106.1 Comparison of ratio of experimental/calculated TEWL.

timeline, rather than as a single irritant exposure as in the previous studies with RA. The results showed a significant synergistic effect with SLS/toluene in tandem regardless of the order of application. We calculated from Wigger-Alberti et al. that TEWL was increased by a factor of 2.9 compared to the expected sum of TEWL of the individual irritants.

A subsequent study analyzed the interaction in a tandem study of 0.5% SLS/60% *n*-propanol.[28] The matched controls in this study were performed with repeated exposures to a single irritant following the tandem application timeline. Measurements were taken for visual scoring, TEWL, and skin color reflectance. The results showed no synergistic effect and limited additive effect with SLS/propanol in tandem studies. Calculating from Kappes et al., we found that TEWL was decreased by a factor of 0.6 compared to the expected sum of TEWL of the individual irritants. It seems that a quenching mechanism rather than an additive one is at work in the interaction between SLS/propanol in tandem irritation studies.

The following study reviewed fruit acids, ascorbic and acetic acid, and NaOH in combination with SLS in tandem.[14] The matched controls were again performed with repeated exposures to a single irritant according to the tandem application timeline. Measurements were taken for visual scoring, TEWL, and skin color reflectance. The results showed that additive effects are seen in all three categories for SLS/ascorbic and acetic acid as well as for SLS/NaOH in tandem studies.

More fruit acid exposures were later investigated in tandem with SLS. Schlemman-Willers et al.[41] investigated citric acid, malic acid, and lactic acid in tandem with 0.5% SLS. The matched controls were again performed with repeated exposures to a single irritant following the tandem application timeline. Measurements were taken for visual scoring, TEWL, and skin color reflectance. Measuring the data published on paper by Schlemman-Willers et al., we found that TEWL was quenched in tandem studies of lactic acid and citric acid, and additive with malic acid. More quenching was observed with the fruit acids at pH 2 rather than at pH 4. No signs of synergistic interaction were seen with any combination in the tandem irritation studies.

106.5 DISCUSSION

The SC, the outermost layer of the epidermis, a lipid protein biphasic structure, is 10–20 μm thick in most surfaces on the human body[36] and consists of two compartments, a desquamating layer of protein-enriched corneocytes embedded in a compact lipid intercellular matrix.[10] The SC poses as the rate-limiting barrier that a compound must penetrate upon entering the skin. As a molecule finds its way into the SC, it is suggested that several pathways are available, through transcellullar/intracellular diffusion, or through shunt pathways through holes or shunts left by hair follicles, glands, skin appendages.[43]

106.5.1 Molecular Size and Hydrogen Bonding

Analysis of the mechanism of penetration on molecules through the SC is valuable for drug delivery research, as well as to assess toxicity to the skin after exposure to a substance.

Another factor of SC penetration by solutes is the maximal delivery flux. It is the maximum dose of a solute able to be delivered over a given period of time and area of application from a given vehicle (J_{max}, mol cm^{-2} h^{-1}).[30] The regression analysis by Magnusson et al. showed that molecular weight (MW) was a dominant determinant of J_{max}, with melting point and hydrogen bonding marginally improving the analysis.[30] J_{max} for solutes with similar MW is associated with a log octanol–water partition coefficient (log K_{ow}) of 2.5–3.0 and greatest for solutes with a low melting point. A separate *in vivo* study by Magnusson showed that a dermal resistance term was unnecessary for *in vivo* predictions for most solutes. Among the solutes studied by tandem irritation, propanol has the lowest molecular weight (MW = 60.1), while toluene has highest (MW = 92.14), yet toluene caused a more significant and synergistic effect with SLS in TEWL. Toluene has a melting point higher than *n*-propanol ($T_{m\ \text{toluene}} = -93°C$, $T_{m\ \text{propanol}} = -127°C$), suggesting that propanol should have the greater flux if T_m was the determinant. Comparing the log octanol–water partition coefficient, toluene has a log $K_{ow} = 2.70$ compared to propanol with log $K_{ow} = 0.34$, indicating their permeation into SC to be primarily controlled by K_{ow}. There is still a need to explore the correlation between solute flux (J_{max}) and clinical TEWL increase *in vivo*. The contribution of K_{ow} on J_{max} appears dominant in the tandem SLS/toluene experiment if a large J_{max} caused the synergistic increase in TEWL.

106.5.2 The Role of SLS as Tandem Irritant

It was previously thought that solvents remove intercellular lipid material resulting in cutaneous barrier impairment. However, subsequent studies show that SLS treatments alter the quality, not the quantity of SC lipids.[21] SLS also play a role in lipid bilayer disorganization,[38] protein denaturation, increased water uptake by SC, and swelling of the corneocytes.[22,47] The increase seen in SC hydration is associated with the uncoiling of an α-helical keratin that unfolds and then incompletely restores with remaining functional defects. These defects are speculated to cause a reduction in water binding capacity causing the clinical symptoms of subsequent roughness and dramatic skin dryness.[47] Pretreatment with SLS in the tandem irritation studies allows for more penetration by a second irritant through mechanical skin barrier impairment. However, the different responses seen in RA/SLS TEWL compared to SLS/RA tandem experiments is an interesting finding that suggests different molecular mechanism of the two irritants. RA appears to decrease skin's susceptibility to SLS effects perhaps by obstructing the percutaneous permeation of SLS.[9]

106.5.3 Tandem Irritants and Percutaneous Penetration

Effendy et al. evaluated the permeation of SLS through human skin after pretreatment with RA, and vice versa, by *in vitro* methods.[8] Cadaveric skin was prepared for measuring the

percutaneous permeation of ^{35}S-labeled SLS and ^{3}H-labeled RA. It was found that an increase in RA penetration was induced by SLS pretreatment; however, pretreatment with RA did not inhibit the percutaneous permeation of SLS significantly compared to untreated skin ($p > 0.05$). This is in contrast to the previous *in vivo* findings of sequential SLS/RA exposure. Other interactions between the irritants and the skin at molecular or cellular levels may play a role in the different skin responses. This study is a start; much remains to be done.

106.5.4 pH Regulation on SC

Exposure of SC to neutralizing buffers and bases has been reported to disturb cutaneous permeability homeostasis.[23] The human skin has long been known to display a protective acidic surface ("acid mantle"), originating from mainly exogenous sources of microbial, sebaceous, and eccrine gland origin. Additional endogenous sources that contribute to acidity include the generation of urocanic acid, the secretory phospholipases from free fatty acids from phospholipids, and the activity of a sodium-proton exchanger.[5]

Upon analyzing the tandem irritation data, we arbitrarily determined our definition of quenching as a tandem TEWL value that is decreased by a factor ≤ 0.5 compared to the expected sum of TEWL of the individual irritants: quenching was observed with lactic acid and acetic acid at pH 2 in tandem with SLS. A proposed explanation could be that the acidic pH allows free fatty acids to assemble more efficiently into ceramides and cholesterol-containing membrane bilayers as proposed by Bouwstra et al.[16] Two enzymes are responsible for this lipid processing, one that hydrolyzes glucosylceramides, and another that hydrolyzes sphingomyelin to ceramides; both are most active at an acidic pH.[16] These mechanisms may contribute to bilayer lipid membrane stability in the tandem studies that utilized SLS/fruit acids at pH 2, as the acidic pH normalizes SC integrity/cohesion, hence the tandem-quenching effect.

106.5.5 Future Studies

The result observed in the tandem irritation studies may shed light on the mechanism of action of the various chemicals as they penetrate the SC. Hypotheses on solute permeation and skin barrier impairment are still investigated from different aspects. The various synergistic, additive, and quenching tandem results may be explained by theories of penetration mechanism and compartment interaction. Penetration is governed mainly by molecular size, polarity, melting point, and H-bond capacity of the permeant; all of which are contributors to the maximum flux of a solute. Compartment interaction depends largely on an active interaction between cutaneous organelles and the permeating solutes. This was observed in the uncoiling of epidermal proteins, alteration in the quality of SC lipids, and activation of SC zymogens as an effect of acidic exposure. More data from tandem irritation experiments will help deduce the pathogenesis of ICD. Measurement with noninvasive bioengineering methods has provided a controlled environment for data collection. Complex interactions between various irritants remain to be solved; hence, special attention is necessary at the workplace and relevant measures should be taken to minimize irritant exposure.

Current information suggests that multiple mechanisms may be involved in the clinical response noted with tandem applications. In spite of this complexity and the intriguing possibility with even more complex chemical mixtures (for instance those in cutting oil), model studies as summarized here provide a difficult but important avenue of investigation to minimize irritant dermatitis. Our hypothesized quenching as suggested by Bouwstra et al. also suggests practical future interventions.

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107 Allergic Contact Dermatitis from Iodine Preparations: A Conundrum?

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107.1 INTRODUCTION

For centuries, iodine compounds have been used as antiseptics and disinfectants [1]. Iodine preparations are available in aqueous solution, tincture of alcohol, aerosol, ointment, antiseptic gauze pad, foam, and swab sticks [2]. Solubility of iodine is 1 g in about 3000 ml water and 13 ml alcohol [3]. In aqueous solution, iodine is present as seven different species: elemental iodine (I_2), hypoiodic acid (HOI), iodine cation ($[H_2OI]^+$), triiodine ion (I_3^-), iodide ion (I^-), hypoiodite ion (OI^-), and iodate ion (IO_3). Elemental iodine, hypoiodic acid, and iodine cation have potent germicidal activity; triiodide and hypoiodite ions are weak oxidants exhibiting mild antibacterial activity, whereas an iodide ion has no antimicrobial activity. Iodate ion has antimicrobial activity only at a pH less than 4, and therefore does not contribute to the germicidal activity of iodine [4]. Povidone-iodine (PVP-I) solution is the most commonly used worldwide because of its potent germicidal activity with relatively low irritancy and toxicity. However, cutaneous irritation has been reported [5–7]. Iodine itself and iodine hydroalcoholic solution are well-known local irritants that can cause iodine burns. To the best of our knowledge, most studies concerning the irritancy of iodine were done either on patients with known iodine-induced contact dermatitis or on normal subjects with PVP-I solution or iodine in ethanol only [6–13]. This study examines the irritant potential and threshold of iodine in three different preparations, namely petrolatum (pet.), 70% isopropyl alcohol (IPA), and PVP-I with different concentrations in normal healthy subjects without iodine allergy, in the hope of refining our knowledge of appropriate nonirritant diagnostic patch-test concentrations.

107.2 MATERIALS AND METHODS

Twenty-four fair-skinned subjects (6 men, 18 women; aged 18–65 years, mean 47 years), in good health and with no history of allergies or significant skin disease, were recruited to participate in this study. The study was approved by the UCSF Committee on Human Research; informed consent was obtained from all subjects. Iodine (99.99% purity) was purchased from Fisher Scientific (Pittsburgh, PA, USA). PVP-I solution 10%, IPA 70%, and pet. United States Pharmacopeia (USP) were supplied by hospital pharmacy. All calculations and compoundings were performed and verified by two registered pharmacists. Finn Chambers (Epitest, Helsinki, Finland) on Scanpor (Norgeplaster, Oslo, Norway) were utilized.

Using closed patch tests with Finn Chambers, concentrations of 0.5, 1, 5, and 10% iodine in pet.; 0.5, 0.75, and 1% iodine in 70% IPA; and 1, 5, 7.5, and 10% of PVP-I were applied to the intrascapular area on the back or to the volar forearm between cubital fossa and wrist as per participants' wish. Purified water was used as a control. The chambers were removed at 2 days (D2). Test sites were read after chamber removal and at 4 days (D4).

107.3 RESULTS

107.3.1 Iodine in Pet.

Most subjects did not react to 0.5% iodine in pet. One subject showed questionable erythema at D2, which had disappeared by D4. Two subjects reacted to 1% iodine in pet. at D2. By D4, an additional three subjects showed mild reactions. With 5% iodine in pet., only four subjects did not react. When

TABLE 107.1
Allergic Contact Dermatitis to Iodine Preparations: A Conundrum

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Gender	F	M	F	F	M	M	F	F	M	F	F	F	M	F	M	F	F	F	F	F	F	F	F	F
Race	W	W	H	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W
Age	52	46	40	54	38	47	49	58	60	43	53	51	32	45	51	51	65	45	18	49	53	65	65	57
I exposed	N	N	N	N	N	Y	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Site	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	A	B	B
I (pet.)																								
0.5% D2	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0.5%D4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1% D2	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	+	0	0	0	0	0	0
1% D4	+	0	0	0	0	0	0	1	0	0	0	0	0	0	+	0	0	+	0	+	0	0	0	0
5% D2	1	0	0	1	2	1	1	1	+	1	1	+	1	1	+	+	0	1	+	1	0	1	0	+
5% D4	1	0	0	+	2	0	2	2	1	1	1	1	0	1	1	+	0	1	2	3	+	2	0	1
10% D2	3	+	1	1	2	1	1	1	1	1	2	3	1	1	1	1	+	1	1	1	1	1	1	1
10% D4	3	1	1	2	3	3	2	2	2	2	3	3	0	1	2	+	+	1	3	1	3	2	1	2
I 70% IPA																								
0.5% D2	0	+	0	0	0	+	+	0	+	+	0	0	0	0	0	0	+	0	+	0	0	0	+	0
0.5% D4	0	+	0	0	0	0	1	0	0	+	0	0	0	0	0	2	+	0	0	0	0	0	+	3
0.75% D2	1	+	0	0	0	+	1	0	1	1	0	0	0	0	0	+	1	0	1	1	1	0	1	+
0.75% D4	1	1	0	0	0	+	0	0	0	1	0	+	3	0	0	3	1	0	0	3	1	0	1	3
1% D2	1	+	0	0	0	+	0	0	+	1	0	0	0	+	0	1	1	0	1	1	1	0	1	1
1% D4	3	3	0	3	0	3	0	3	0	2	0	3	0	3	0	3	1	0	3	3	3	3	1	3
PVP-I																								
1% D2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1% D4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5% D2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5% D4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7.5% D2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7.5% D4	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
10% D2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
10% D4	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
H_2O	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

F = female; M = male; W = white; H = hispanic; B = back; A = arm; I = iodine; pet. = petrolatum; IPA = isopropyl alcohol; and PVP-I = povidone-iodine. Test site was the back in 23 of 24 individuals and 23 of 24 were Caucasians. Six were males. Subjects that were negative to all concentrations are not shown. Subject 6 was the only individual known to have repeated iodine exposure. Negative (not shown) were I (pet.) 0.5% on day 4; PVP-I 1 and 5% on days 2 and 4, 7.5% on day 2, and 10% on day 2; and H_2O (control) on days 2 and 4. Skin reactions were graded according to the following scheme: 0 = no reaction; + = questionable erythema; 1 = definite erythema; 2 = erythema and induration; and 3 = vesiculation.

TABLE 107.2
Patch-Test Reactions to Iodine Preparations

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Age	52	46	40	54	38	47	49	58	60	43	53	51	32	45	51	51	65	45	18	49	53	65	65	57
I (pet.)																								
0.5% D2	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1% D2	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	+	0	0	0	0	0	0
1% D4	+	0	0	0	0	0	0	1	0	0	0	0	0	0	+	0	0	+	0	+	0	0	0	0
5% D2	1	0	0	1	2	1	1	1	+	1	1	+	1	1	+	+	0	1	+	1	0	1	0	+
5% D4	1	0	0	+	2	0	2	2	1	1	1	1	0	1	1	+	0	1	2	3	+	2	0	1
10% D2	3	+	1	1	2	1	1	1	1	1	2	3	1	1	1	1	+	1	1	1	1	1	1	1
10% D4	3	1	1	2	3	3	2	2	2	2	3	3	0	1	2	+	+	1	3	1	3	2	1	2
I 70% IPA																								
0.5% D2	0	+	0	0	0	+	+	0	+	+	0	0	0	0	0	0	+	0	+	0	0	0	+	0
0.5% D4	0	+	0	0	0	0	1	0	0	+	0	0	0	0	0	2	+	0	0	0	0	0	+	3
0.75% D2	1	+	0	0	0	+	1	0	1	1	0	0	0	0	0	+	1	0	1	1	1	0	1	+
0.75% D4	1	1	0	0	0	+	0	0	0	1	0	+	3	0	0	3	1	0	0	3	1	0	1	3
1% D2	1	+	0	0	0	+	0	0	+	1	0	0	0	+	0	1	1	0	1	1	1	0	1	1
1% D4	3	3	0	3	0	3	0	3	0	2	0	3	0	3	0	3	1	0	3	3	3	3	1	3
PVP-1																								
7.5% D4	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
10% D4	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

I = iodine; pet. = petrolatum; IPA = isopropyl alcohol; and PVP-I = povidone-iodine.

increased to 10% iodine in pet., all 24 subjects reacted. Most developed erythema at D2 and induration or vesicles by D4 (Table 107.1).

107.3.2 Iodine in 70% IPA

When adding iodine to 70% IPA, a 0.5% concentration elicited questionable erythema in eight subjects at D2, with stronger reactions seen in three subjects. With the 0.75% concentration, more than half of the subjects had questionable erythema to definite erythema at D2. Four subjects had vesicles by D4.

107.3.3 PVP-I and Purified Water

One subject developed reactions to 7.5 and 10% PVP-I at D4. None had any skin reactions to purified water (Table 107.2).

107.4 DISCUSSION

Iodine 0.5% in pet. was almost nonirritant. Only 1 of 24 subjects exhibited a questionable skin reaction, which had resolved by D4. Its irritant threshold appears to reside near 1% concentration, as evidenced by 21% of the subjects having mild skin reactions at D4. Iodine 5 and 10% in pet. are definitely irritant. Almost all subjects had some reactions, with over 37% of the participants demonstrating vesicles. Pet. may increase the irritant potential of iodine by increasing its contact with the skin surface. Five subjects did not react to all concentrations tested of iodine in 70% IPA, whereas others demonstrated significant reactions to the 1% concentration at D4. Both alcohol and iodine are acute irritants. The combination of alcohol with iodine may have additive irritant effects. Alcohol removes sebum from the skin surface, which might enhance the penetration of iodine causing more irritation. PVP-I solution equal to or less than 10% concentration proved to be the least irritant among the preparations tested. Only one subject had a reaction at D4. PVP acts as an effective iodophor, which complexes with iodine in aqueous solution. A 10% PVP-I solution contains 10% bound iodine and 1% available iodine, making it less toxic and irritant, without reducing its antiseptic properties [11]. Overall, iodine must be patch tested with caution. Considerable individual variation was noted. A larger population would presumably demonstrate even greater variation. In this clinical situation, careful review of various steps in the operational definition of allergic contact dermatitis seems to be appropriate when correlating patch-test response with clinical background. We note that only in Refs. 1, 11, and 12 were subjects patch tested with what now appear to be nonirritant preparations. We suspect that much of these reports represent irritation rather than allergy.

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108 Human Skin Buffering Capacity: Overview

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108.1 INTRODUCTION

When dilute acids or alkalis contact the skin surface, the induced change in pH is temporary and the original acid reaction is rapidly restored, meaning that when an acidic or alkaline solution contacts skin the H^+ ion or the OH^- ions, respectively, disappear. What happens to these ions?

Possibilities include the following:

First, the epidermis is a gel and therefore it cannot be excluded that OH^- ions are adsorbed into the colloidal system. In adsorption in a colloidal system, anions and cations will disappear but not in the same ratio as an interchange of ions will take place.

The second possibility is the absorption of base, such as NaOH, in intact skin. This occurs with salicylic acid in which case keratolytic properties of the drug are important [69]. Alkali causes swelling of keratin; hence, the possibility of absorption deserves further study. In the case of absorption, corresponding cations and anions will disappear from the solution.

A third possibility is the neutralization of OH^- ions by H^+ ions in which case the number of anions decreases but the number of cations in solution does not change.

Therefore, the amount of cations in the solution in contact with the skin gives important indications on the mode of neutralization. It was noted that a 0.1 N NaOH and a 0.1 N KOH solutions did not show a decrease in cations compared with a sample of the same solution that had not been in contact with the skin, implicating that the neutralization of low-titer alkali solutions by the skin is caused by chemical reaction with an acid buffering system on the skin's epidermis [68].

108.2 WHAT IS A BUFFER?

A buffer is a substance that minimizes change in the acidity of a solution when an acid or a base is added to the solution or is diluted. Buffer solutions consist of a weak acid and its conjugate base. Buffers resist changes in pH by resisting

changes in the hydronium ion concentration. This resistance is the result of the equilibrium between the weak acid (HA) and its conjugate base (A^-) (i.e., the buffering system):

$$HA(aq) + H_2O(I) \rightleftharpoons H_3O + (aq) + A^-(aq)$$

With this buffering system, any alkali added to the solution is consumed by hydronium ions. The hydronium ions are then mostly regenerated as the equilibrium moves to the right and some of the acid dissociates into hydronium ions and the conjugate base. If a strong acid is added to the buffering solution, the conjugate base is protonated, and the pH (i.e., hydronium ion concentration) is almost entirely restored. This is an example of Le Chatelier's principle and the common ion effect [72]. This contrasts with solutions of strong acids or strong bases, where any additional strong acid or base can greatly change the pH.

Buffer systems can be represented as salt of conjugate base/acid, or base/salt of conjugate acid.

The acid dissociation constant (K_a) is a specific type of equilibrium constant that indicates the extent of dissociation of hydrogen ions from an acid (i.e., how well it splits H^+ ions). K_a differs for each acid and varies over many degrees of magnitude; hence, the constant is often represented using the same mathematical relationship as $[H^+]$ is to pH and is represented by the symbol pK_a.

$$pK_a = -\log_{10} K_a$$

pK_a is inverse of the common logarithm of K_a.

In general, the larger the value of K_a (or smaller the value of pK_a) the stronger the acid is, meaning that the extent of dissociation is larger at the same concentration.

A buffering system's pK_a represents the pH where 50% of buffer is dissociated, which is also indicative of the optimum pH buffering range. The optimum buffering range occurs ± 1 pH units from the pK_a [12].

108.3 WHAT DO WE MEAN BY "BUFFER CAPACITY" OF THE SKIN?

The acidic character of skin was first mentioned by Heuss [27] and by Schade and Marchionini [51] who introduced the term "acid mantle" to describe the skin's acidic character. The importance of skin's acidic character has more recently been discovered as playing a crucial role in barrier homeostasis and immune function [22,24,30]. The acidity of the skin surface can be measured according to two criteria: its value given by the pH, and its strength determined by the ability of the skin to resist an acidic/alkaline aggression (i.e., acidic/alkaline resistance and neutralization tests) [1]. It is the quantitative extent to which the skin resists these changes that defines the term buffer capacity or acid/alkali resistance and neutralization capacity of skin. This chapter reviews studies investigating the acidic nature of skin including a brief summary of experimentally determined skin pH values and a more in-depth investigation of the mechanisms contributing to the buffering capacity of the epidermis via alkali/acidic aggression tests. This chapter will discern which components of the epidermis are most likely responsible for the buffering ability of the epidermis.

108.4 MEASURING SKIN ACIDITY

108.4.1 Reported pH Values

The skin pH is a measure of the H^+ concentration in the aqueous solution present on the skin surface. The method universally used today is the glass planar electrode (potentiometric method). This method has replaced the colored indicators (colorimetric methods), which were half as accurate and could give erroneous results, as well as the method based on the hydrogen electrode (electrometric method) which is more cumbersome to use. The glass planar electrode is based on the difference in potential created on each side of a thin glass slide separating two solutions, which is linearly linked to their difference in H^+ concentration. The other advantage of the planar surface of the electrode is its excellent contact with the skin [1].

Skin surface pH can be influenced by numerous endogenous and exogenous factors. Endogenous factors can be related to clinical pathological situations such as the characteristic increase in skin pH in atopic dermatitis or unrelated to pathological factors such as the changes in pH with age, anatomical location, or gender. Examples of exogenous factors that influence pH include an individual's personal care product routine and their perspiration rate. Table 108.1 lists elements that affect skin pH.

Table 108.2 is a collaboration of reported skin pHs from studies sorted by year of publication. Keeping in mind that pH is a logarithmic scale, the skin pH values reported show a high degree of variation between both the pH of individual subjects within each study as well as between the studies themselves. Yet the general consensus using the latest technology is that skin pH falls in the range of 4–6 (± 0.2).

The wide range of pH values reported may be influenced by intra- and inter-individual variations. Causes of intraindividual variation include the nonpathological endogenous and exogenous influences on an individual pH mentioned above. For example, the extent of SC hydration will vary within an individual depending on the change of the seasons as well as their personal care product routine. Speculating potential causes of interindividual variation, one must consider the influencing factors for skin pH such as genetic predisposition and thickness of the horny layer.

Recent experiments attempt to minimize exogenous intra- and inter-individual variations before experimentation by prohibiting the use of personal care products 24 h prior to measuring the skin pH. Although this is a step in the right direction, the subjects should also refrain from washing with plain tap water which typically has an alkaline pH and as

TABLE 108.1
Elements Affecting Skin pH [72]

Physiological
Age
Anatomic site
Genetic predisposition
Race (ethnicity)
Sebum
Stratum corneum hydration
Sweat
Exogenous
Antibacterials (topical)
Cosmetic products
Detergents
Occlusive dressings
Soaps
Skin irritants
Pathological
Atopic dermatitis
Diabetes mellitus
Diaper dermatitis
Irritant and allergic contact dermatitis
Uremia

TABLE 108.2
Skin pH Reported Sorted by Year Published

Study	pH Reported	Year Published	Method
Sharlit and Sheer [58]	5.5	1923	Color indicators
Schade an Marchionini [52]	3–5	1928	Electrometric methods
Herrmann and Furst [26]	3–5	1929	Colorimetric study
Blank [4]	4.2–5.6	1939	Potentiometric
Schmid [54]	4.8–5.8	1952	Color indicators and potentiometric methods
Schirren [53]	4.2–5.6	1955	Potentiometric glass electrode and quinhydrone electrode[a]
Green and Behrendt [20]	4.5–5.8	1971	Potentiometric
Braun-Falco and Korting [5]	5.4–5.9	1986	Potentiometric
Zlotogorski [74]	4–4.9	1987	Potentiometric
Korting et al. [32]	4–4.9	1987	Potentiometric
Dikstein and Zlotogorski [13]	4.5–6.0	1994	Potentiometric
Parra and Paye [46]	4–6	2004	Potentiometric
Jacobi et al. [29]	4.2–6.0	2005	Potentiometric
Lambers et al. [33]	4.0–5.0	2006	Potentiometric

demonstrated will result in a more alkaline skin pH [33,45]. Lambers et al. [33] assessed the skin surface pH of the volar forearm before and after refraining from showering and cosmetic product application for 24 h. The average pH dropped from 5.12 ± 0.56 to 4.93 ± 0.45 ($N = 330$). On the basis of this pH drop, Lambers et al. concluded that the "natural" skin surface pH is on average below 5.0. This is in contrast to the general assumption that skin surface pH ranges between 4.0 and 6.0 (Table 108.2).

The variation in skin pH values presented in Table 108.2 could also be due to the differences in experimental metodology, equipment used, the type and number of subjects picked for the study, and the site(s) chosen for anatomical experimentation. The influence of methodology was mentioned previously in Section 108.4.1. When selecting subjects for experimentation, the influence of certain factors such as gender and age of the subjects on skin pH should be kept in mind as there is a significant difference between the pH of males and females in certain anatomical locations [16,22,47] and skin pH generally increases as an adult ages [20,47,64,70]. In addition, due to the established topographic pattern of skin pH, when reporting skin pH it should be noted which anatomical location was used to measure the skin pH and whether more than one anatomic location was used to calculate an average. Because of the influence of personal care products and water, it should also be noted how long the subjects have refrained from any product or water use prior to experimentation.

In addition, intertriginous areas (axilla, genitoanal, and interdigital regions) and the regions supplied by apocrine glands should be regarded as a separate category because these areas are less acidic than the rest of the body and have distinguishing characteristics [55].

It seems impossible to discern a more accurate (precise) skin pH range for healthy adults without reporting pH values by anatomic location, gender, age, and other distinguishing skin characteristics because of the endogenous and exogenous factors that affect skin pH.

Therefore, it is for simplistic reasons that authors report a wide pH range, to account for the influence of many of the endogenous and exogenous factors on skin pH.

It is hard to ignore the conclusion of Lambers et al. [33] that the healthy/natural skin pH is below 5, as it is in accordance with the recent research concerning skin's optimal functioning at a pH below 5.0. It is demonstrated that skin with pH values below 5.0 is in a better condition than that with pH values above 5.0, as shown by measuring the biophysical parameters of barrier function (integrity and cohesion), moisturization, and wound healing [23,25,37,38,47]. The effect of pH on adhesion of resident skin microflora was also assessed; an acid skin pH (4–4.5) keeps the resident bacterial flora attached to the skin, whereas an alkaline pH (8–9) promotes the dispersal of normal flora from the alkaline skin sites and hence will allow the colonization of pathologic flora [31,33,55,65]. It is clear that further research is needed to substantiate this theory.

108.4.2 Alkali/Acidic Aggression Tests

An acid/alkali aggression test is another way to measure the acidity of skin, more specifically the acid/alkali resistance and acid/alkali neutralization test. Alkali/acidic resistance refers to the maximum allowable concentration and amount of acid/alkali applied before corrosion of the skin. Acid/alkali resistance tests were more commonly used in the 1960s to detect workers that were likely to develop occupational diseases in certain chemical work environments [1]. Acid/alkali neutralization tests assess how quickly the skin is able to buffer applied acids/bases. Repetitive applications of acid or base demonstrate that it is possible to occupy/consume/overpower the buffering ability of the skin and hence increase the time required for neutralization [6–9].

The next section focuses on the aggression tests specifically designed to uncover what components of the epidermis are responsible for the buffering capacity or neutralization capacity of skin.

108.5 COMPONENTS OF THE EPIDERMIS THAT POTENTIALLY CONTRIBUTE TO THE BUFFER CAPACITY (AND NEUTRALIZATION ABILITY) OF THE SKIN

108.5.1 Free Fatty Acids (FFA)/Sebum

The sebum is skin's first defense against alkali/acidic application. McKenna [39] and coworkers examined the composition of the sebum by exposing the hand and forearm to acetone and chloroform. They found that this "skinfat" contained about 1% nitrogen, for the greater part in urea and the rest in ammonia and as lipid nitrogen. They found no amino acids (AA) in the skin fat. Despite the lack of AA in sebum, the acidic nature of the fat could contribute to alkali neutralization. Experiments to date suggest that sebum plays a negligible role in the neutralization of both acids and alkalis.

Lincke [34] was one of the first to question whether the normal fatty acids on the skin surface play a role in buffering. He found that sebum had no acid-buffering capacity at all and had an extremely weak buffering effect on alkalis with the maximum effect at pH 9 where $3–5 \times 10^{-4}$ mol alkali is neutralized by 1 g of fat. This would amount to the neutralization of 12–20 mg of NaOH by 1 g of sebum. That much buffering appears negligible.

According to Dunner [14], sebum influences the neutralization of alkali in two ways: first, it protects the epidermis against the influence of alkali by slowing down the exposure and penetration of acids or alkalis applied to the skin [14,15,66]. Second, the fatty acids in sebum could have importance for the alkali-neutralization capacity of the skin. Lincke, as mentioned above, concluded that fatty acids only play a minor part in alkali neutralization [68].

Through experimentation, Dunner observed a quicker neutralization on skin where lipids had been extracted beforehand, than on a corresponding nontreated area [14,66,68]. Vermeer came to the same conclusion using his own method of alkali neutralization. He compared the neutralization on the soles, on the forearm, and on a corresponding area of a forearm after sebum removal [68]. Utilizing the palm and sole for comparative purposes contributes two variables: the lack of sebum and a thicker stratum corneum (SC). Separating these two effects has yet to be accomplished.

Today most neutralization experiments are performed after cleansing the skin with lipid solvents, which remove most of the fatty acids. This is done because it is generally accepted that the sebum plays a negligible role in the neutralization of alkali and acids and invariably neutralization proceeds faster when fat is removed [14,15,49,66,68]. Nevertheless, lipid solvents use acids, an artifact not yet adequately understood.

Vermeer [66] and Neuhaus [41] believe that the increased speed of neutralization is due to the greater carbon dioxide (CO_2) diffusion. This theory, discussed later in detail, is generally not accepted and not clearly substantiated either way. Vermeer et al. [68] discovered that after lipid removal more acid was produced by the skin in all trials, which could account for the faster neutralization. The same group also found that the increase in neutralization caused by the lipid removal was limited to the first quarter of an hour. This limited increase in neutralization is possibly because the sebaceous glands had rebuilt a sufficient layer of sebum, but whether the acid secreted after lipid removal was a secretion to rebuild the lost sebum layer or another such constituent to contribute to the faster neutralization was not tested. Either way it seems that the lipid in sebum slows down the penetration of acids/alkalis on to the skin and its removal allows for faster neutralization whether it is due to greater secretion of acid or more exposure of the applied substance to other water-soluble components of the epidermis responsible for the buffering capacity of skin.

It is pertinent to note for future experiments, which do not remove the sebum before an alkali/acidic aggression test, that while the sebum excreted by the sebaceous glands is spread out in an even layer on the surface of the skin [11], the amount of sebum secreted, its constituents, and viscosity vary with anatomic location, body temperature, and subject's age (i.e., hormonal influence) [10,21,66]. It is the opinion of this review that the contribution of the sebum is negligible to the buffering capacity of the skin and that removal of the sebum before experimentation may result in more reproducible results and decrease the level of inter- and intra-individual variations. Extension of experimental data should confirm or deny this hypothesis.

108.5.2 Epidermal Water-Soluble Constituents

Vermeer et al. [68] demonstrated the importance of water-soluble constituents to the skin's buffering ability. By soaking skin in water, the water-soluble constituents were removed from the surface and from within the horny layer, and the neutralization capacity was greatly reduced [68]. Hence, constituent(s) of the water-soluble substance in the horny layer of skin is/are major contributors to the buffering capacity of

the skin [8,56,68]. Undoubtedly, water soaking may produce other changes that have yet to be accounted for.

The significance of water-soluble constituents of the epidermis to the buffering capacity of skin further supports the theory of minimal contribution from the sebum of skin due to its lipid-soluble nature [68].

108.5.3 Sweat

It is indisputable that eccrine sweat initially accelerates the neutralization of alkalis [6–9,67,68,71]. Yet the question remains as to what component of sweat is responsible for the increased buffering capacity. Spier and Pascher [59] suggest that the main components of sweat to consider are lactic acid and AA.

The lactic acid–lactate system, provided by sweat, has a highly efficient buffering capacity at pH 4–5 [15]. Yet we are unaware of any experiments that demonstrate lactic acid to be the main buffering agent in sweat or the skin surface.

Conversely, the role of AA as a buffering agent of sweat and surface of the horny layer has been investigated thoroughly. Vermeer et al. [68] showed that after alkaline solutions in contact with the defatted human skin surface contain AA; these authors believed that the principal factors in buffering the surface are the amphoteric AA secreted by the sweat glands. Wohnlich [71] too ascribed the greatest importance to sweat in which AA are dissolved as a result of contact with keratin.

After discovering the presence of AA in eccrine sweat and the skin surface, Vermeer et al. [68] compared the role of AA in neutralization of a person sweating versus a person that is not sweating. In both instances (sweating and not sweating) they found that AA play a significant role in neutralization during the first 5 min. When comparing the formol titration with phenolphthalein titration, the formol values increased in the presence of eccrine sweat while the phenolphthalein values remained the same. Since the formol values only represent the amount of AA present while the phenolphthalein values represent all the acids present (including lactic acid), this is a case to support that it is the AA in sweat that contribute to the faster neutralization of skin.

108.5.4 Keratin

The contribution of keratin to the buffering capacity of skin remains in question.

Keratin is an amphoteric protein with the ability to neutralize acids and alkalis *in vitro* [6–9,28,34,43,58] and hence may buffer any reaction on the skin surface. Scales scraped from normal skin do bind to small amounts of alkali *in vitro* [35,60].

If SC keratin filaments contribute to the buffering capacity of the SC then according to Vermeer et al. [68], keratin's amino-end cation (NH_3^+) would bind to the alkali (OH^-) and hold the alkali on the skin making it disappear from solution. Instead it is known that the majority of the alkalis applied to the skin are neutralized in the water-soluble solution of the epidermis and remain in solution. In addition, Vermeer et al. confirmed that the majority of the buffering capacity of the skin comes from the water-soluble constituents of the epidermis, not the insoluble constituents such as keratin.

Although insoluble keratin filaments on the skin, unlike hair, may not have buffering capacity [20,68], it is possible that the breakdown of keratin into its constituent AA contributes to the free AA of the water-soluble portion of the epidermis. In that case we would expect the free AA in the water-soluble portion of the epidermis to be similar in composition to the AA in keratin.

Peterson and Wuepper [17] isolated the SC keratin and found high numbers of glutamic acid, glycine, and proline and smaller amounts of serine and cysteine depending on the type of keratin isolated (confirmed by Goldsmith [18]). Yet, serine, aspartic acid, citrulline, and arginine were shown by Spier and Pascher [59] to be the main components of the water-soluble portion of the startum corneum. If keratin was a major contributor to the pool of free AA, it would be expected to find high levels of glumatic acid and glycine. Whether glutamic acid and glycine are incorporated into other epidermal constituents or penetrate to lower levels is unknown.

Still, despite the lack of concrete evidence to support keratin's contributing role to the buffering capacity of the epidermis, a modifying action of keratin is assumed [34]. Of course without an intact keratin layer, neither a physiological surface pH nor normal neutralization capacity can be maintained [2].

Further research needs to be completed to determine if keratin's role directly contributes to the buffering capacity or indirectly contributes by adding to the pool of free AA in the water-soluble portion of the epidermis.

108.5.5 Horny Layer Thickness

Individual differences in the acid-binding capacity of skin is fairly uniform, yet there are characteristic individual differences which may be due to differences in thickness of the horny layer [49].

The thicker the horny layer, the better the buffering is [8,68]. This might be due to a greater storage of water-soluble buffers within a thicker horny layer [49]. This makes sense when you consider what happens to aging skin. As the skin ages, its thickness and buffering ability diminishes [70]. It is possible that the diminished buffering ability of aged skin is a result of the thinning of the horny layer. Current technology permits more accurate SC; mensuration should aid clarification of this point [57].

108.5.6 CO_2

Little is known about the CO_2/HCO_3 system on the epidermis. Burckhardt's studies were the first to suggest that the CO_2 diffusing from the epidermal layer is responsible for neutralizing alkali that is in contact with the skin [6–9]. Burckhardt's experiments demonstrated [6,7] that if a 5-min alkali-neutralization experiment is repeated subsequently several times in the same area, the later neutralization times are longer

than the earlier ones but they finally become constant. His interpretation was that in the beginning the fixed acids on the skin surface bind the alkali, but later on, it is the steady diffusing CO_2 which neutralizes the alkali. This view was accepted by Piper [43], Szakall [62,63], and Neuhaus [41].

Piper [43] also hypothesized the role of CO_2 in the neutralization of alkali and concluded that neutralization is partly due to the diffusion of CO_2 to the skin surface. He found that if a tube filled with a fixed alkali solution is placed on the skin, after a while there is no more uptake of amphoteric buffering agents from the skin surface. Piper believed that the diffusion of CO_2 increases to continue to buffer the applied alkali.

Szakall and Neuhaus [62,63] experimented with conventional soaps and neutral detergents which defatted skin surface. Szakall attributed the increased speed of neutralization after removal of lipid on the skin surface to the greater diffusion of CO_2. Szakall also noted the importance of the state of hydration of the horny layer after rinsing, because he postulated that the hydrated horny layer retains CO_2 and slows down its diffusion. Thereby, Szakall noted that moderate hydration is desirable, promoting maintenance of the acid reaction [63].

These experiments present quantitative data on neutralization capacity of skin but fail to provide quantitative support for their conclusions concerning CO_2. For example, Burckhardt completed repetitive neutralization experiments and presented quantitative data revealing that after repeat application of alkali, neutralization time eventually becomes constant. Yet there is no quantitative evidence suggesting that the reason for the constant neutralization times is CO_2. Burckhardt's conclusion concerning CO_2 was a speculation. Other studies attribute the eventual constant neutralization time to the destruction of the "skin barrier" and unlimited penetration of the applied alkali [41,68]. Szakall's conclusions should be considered in the same fashion as Burckhardt's. In Szakall's experiments [62,63] there are quantitative data recording the accelerated neutralization time after defatting the skin surface, yet his explanation of the accelerated neutralization time as a result of increased CO_2 diffusion is not backed by quantitative data. Piper provided the only experiment to quantitatively measure the increase in the amount of CO_2 in a closed system. But whether this increase in CO_2 was responsible for the neutralization in the second half hour was never totally substantiated.

Vermeer et al. [68] conducted thorough experiments to determine which acids were principally responsible for the alkali neutralization by the skin. Knowing that several authors (Piper, Szakall, and Neuhaus) considered the production of CO_2 of great importance for alkali neutralization but did not have any concrete quantitative data for such a hypothesis, Vermeer et al. modified their experimental design to determine the quantitative contribution of CO_2 to the neutralization of alkali. In their original experimental design, the alkali solutions were regularly shaken in close contact with the skin and also mixed continually with air. The uncontrollable quantity of CO_2 from the air would render any quantitative analyses of CO_2 unreliable. Hence, they eliminated the CO_2 entirely by adding an overmeasure of HCl after titration with phenolphthalein. Phenolphthalein titrations will account for any number of water-soluble acids, including AA, lactic acid, and bicarbonate. Addition of the HCl will decompose any CO_2 accounted for in the titration and will allow it to escape. The solution was then boiled and cooled by running water to eliminate any remaining CO_2 in the solution. Then, the overmeasure of HCl was titrated with 0.1 N NaOH. The low values obtained by titration after adding an overmeasure of HCl make it improbable that CO_2 is of great importance [68]. These calculations gave the amount of alkali neutralized by the skin, eliminating the possible neutralization by CO_2 produced by the skin.

In the above experiment, Vermeer et al. [68] paid attention to what was happening in the first minutes of the neutralization process, whereas Piper [43] followed the events up to 1 h. Piper concluded that, for half an hour, alkalis are neutralized on the skin by the skin's own amphoteric substances (such as AA), but that in the second half hour diffusing CO_2 takes over. Piper's conclusions are not necessarily contradictory to the results obtained by Vermeer above and may actually be in agreement. According to Piper, "the longer the contact between skin and alkali lasts, the greater the importance of CO_2 will be." Further quantitative studies need to be completed to substantiate or refute this hypothesis.

More recent experiments concerning the role of CO_2 as buffering agent of skin are sparse, but there are recent case reports demonstrating the effectiveness of CO_2-impregnated water on diseased skin. These case reports prompted Rieger [44] to conduct a more thorough investigation concerning the influence of CO_2-impregnated water on the skin. In the case study, 102 hairdressers who regularly applied CO_2-impregnated water on their diseased skin (mostly eczema) were questioned. Of these, 76% reported a favorable influence of application on their skin disease. In the experiment, a unilateral-controlled study was conducted to objectively determine the possible preventative and therapeutic effects of CO_2-impregnated water on experimentally induced skin damage. The effect of CO_2-impregnated water on cumulatively irritated skin was assessed by measuring transepidermal water loss (TEWL), the conductivity, microcirculation (laserflow Doppler flowmetry), and SC pH. The results showed that CO_2-impregnated water favorably influenced the physiological parameters in cumulatively irritated skin, including pH. Even by clinical assessment positive effects of CO_2 application could be found, compared to the test area with normal water as a control [44].

108.5.7 Neutralization of Alkali $Ca(OH)_2$

Vermeer et al. [68] investigated the neutralization of a different alkali—$Ca(OH)_2$. The results of its phenolphthalein titration and formol titration were compared to the titration values of previous NaOH experiments. The $Ca(OH)_2$ neutralization resulted in higher phenolphthalein titration values. Remember that formol titration values represent the amount of AA present, while phenolphthalein values

are inclusive of other acids that may be present, such as lactic acid and carbonate. The higher phenolphthalein values therefore imply that acids other than AA play a more significant role in the neutralization of $Ca(OH)_2$. This may be because Na_2CO_3 is soluble, whereas $CaCO_3$ is insoluble. Moreover, the Ca salt of fatty acids is a plaster by which most probably a part of the $CaCO_3$ is fixed on the skin. Consequently, they cannot, as in experiments with NaOH, neglect CO_2 as a factor in the neutralization. The colorimetry and formol values for the $Ca(OH)_2$ experiment run parallel, but the colorimetry values run at a lower level. The difference of the colorimetric values in the $Ca(OH)_2$ and the NaOH experiments does not prove that the amount of AA involved in neutralization is less; the possibility remains that $Ca(OH)_2$ and NaOH are neutralized by different AA. Interesting, but nonconclusive. This experiment puts back into play the idea that CO_2 may play a role with some bases, as well as that different AA may be involved in the neutralization of different compounds [68].

Additional consideration should be kept in mind in aggression tests with Ca-containing compounds. Acute barrier disruption by organic solvent treatment, detergents, or tape-stripping results in the passive loss of calcium from the upper epidermis and hence disruption in the calcium gradient [16,36,40]. It is this disruption in the Ca gradient that accelerates both lamellar body secretion and lipid synthesis [40]. Hence, consider that the application of Ca may delay lamellar body secretion and lipid synthesis hence affecting barrier homeostasis and any barrier repair, and therefore may utilize different mechanisms than when other non-Ca-containing alkalis are applied.

In summary, there seems to be a consensus that CO_2 does not contribute to the initial alkali neutralization process. This is also supported by the recent discoveries of relatively low level of oxygen consumption, CO_2 production in the epidermis, and only limited activity of the Kreb's cycle suggesting that a minimal amount of CO_2 would be available for neutralization [17]. But whether CO_2 contributes to the neutralization after significant exposure of an alkali, once all the amphoteric buffering agents are utilized, or in the absence of buffering agents as in skin disease is a hypothesis that has yet to be supported or refuted by quantitative data.

108.5.8 Free Amino Acids

The free AA in the water-soluble portion of the epidermis play a significant role in the neutralization of alkalis within the first 5 min of experimentation [28,43,68].

Piper [43] found good buffering capacity from pH 4 to 8 with optimum of 6.5 ($pK_a = 6.5$), which again speaks more for the decisive role of AA than of the lactic acid (which has a more acidic pK_a of 4–5). He calculated in one case the dissociation constant of an acid mixture delivered to the skin to be $K = 2.95 \times 10^{-5}$, in the range of constants of carboxylic acids in general [49].

In agreement with Piper, Jacobi [28] found if the amino groups of AA and proteins are bound by formaldehyde or by nitrous acid, both the acidity of the surface and the alkali-neutralization capacity increase indicating once again the role of carboxylic groups of AA.

After discerning that the neutralization of alkali cannot be explained by the presence of fatty acids, Vermeer et al. [68] turned their attention to AA. After removing sebum, they applied an alkaline solution to the skin and collected this alkaline solution to determine the amount of AA present by formol titration. Formol titration is a method of titrating the amino groups of AA. By adding formaldehyde to the neutral solution, the formaldehyde reacts with the NH_3 group, liberating an equivalent quantity of H^+, which may then be estimated by titration with NaOH. Besides the formol titration, the presence of AA was demonstrated with the ninhydrin, B-napthol quinone sulfonate, and Nessler's test. Nessler's test was used to ascertain that no ammonia was present as ninhydrin and B-napthol quinone sulfonate also reacts with ammonia salts. No ammonia was found. Vermeer et al. [68] assumed that the amount of formol added as well as the amount of additional NaOH added represented the total amount of AA present, which later they determined to be a mistake, as the values obtained by the phenolphthalein titration also include other acids present, not just AA. In their method, CO_2 is eliminated but other less volatile acids are not excluded and may slightly contribute to their quantitative calculation of AA from the phenolphthalein titration presented in Table 108.3 (column 4).

Vermeer et al. [68] also repeated the experiment without using a lipid solvent and came to the same conclusion—AA are responsible for neutralization within the first 5 min shown by the peak in formol values. To calculate the amount of AA from the results of the titrations, the molecular weight must be known, and for calculation of the results of the colorimetric determination, the number of nitrogen atoms in the amino acid molecule must also be known. It is not likely that we are dealing with just one AA. Rothman and Sullivan found 11 different AA in water that have been in contact with the skin [50]. Assuming that the average molecular weight (MW) of AA is 140 and that they possess only one N atom, and neglecting the possible influence of lactic acid and perhaps other acids, Vermeer et al. calculated the amount of AA (Table 108.3).

The values calculated from the titrimetric method are higher than those from colorimetry, the difference being largest during the first 5 min. It has already been pointed

TABLE 108.3
Vermeer et al. [68] Calculate the Amount of Free Skin AA Involved in Buffering Alkali Solutions

Portion (Every 5 Min)	Phenolphthalein Titration	Formol Value	Calculated AA in mg (Titrimetr.)	Calculated AA in mg (Colorimeter)
1	3.6	1.56	72	48
2	1.59	1.08	37.5	29.4
3	0.96	1.14	29.4	23.8
4	0.84	0.93	24.8	27
5	0.54	0.75	18	10
6	0.27	0.81	15	18

out that the presence of lactic acid and other acids offers a possible explanation.

Despite the general agreement about the role of AA in the neutralization of alkalis, we know little about which AA are the key buffering agents. It is likely that more than one AA is responsible for the skin's buffering ability, but it is not confirmed yet. Fortunately, the AA composition of the upper SC was reported by Spier and Pascher [59].

Spier and Pascher reported that the free AA of the SC account for 40% of the water-soluble substances extracted from this SC by analysis of graded skin strippings [20,59]. Of the AA present, 20–32% was serine and 9–16% was citrulline. Aspartic acid, glycine, threonine, and alanine were 6–10%. The smallest concentration of AA was glutamic acid at 0.5–2%. It is surprising that there is not a significantly larger number of dicarboxylic acids such as glutamic and aspartic acid on the skin, since there was speculation that carboxylic acid of the amino acid plays a significant role in the skin buffering capacity [59].

The origin of water-soluble free AA on the skin surface may be from three plausible different sources:

1. Eccrine sweat

Sweat contains 0.05% AA which remain on the skin surface after evaporation. The specific AA found in sweat was not investigated.

2. Living epithelium

As discussed earlier, keratinization and the degradation of desmosomes may be a source for AA such as serine, glycine, and alanine.

3. Hair follicle

Citrulline is recognized as a constituent of protein synthesized in the inner root sheath and medulla cells of the hair follicle. Specific proteases release citrulline and it becomes free. Citrulline was also found in proteins in the membrane of the SC as well as free floating by Steinhert and Freedberg [61].

The AA responsible for buffering capacity may be dependent on what is available at that moment, on the applied alkali/acid, or on the activity of a specific metabolic pathway responsible for making available certain AA that are responsible for neutralization.

Either way, further research needs to be completed to discover which AA contribute to the buffering capacity of skin and what is the main source of these AA.

108.6 DISCUSSION

108.6.1 Influence of the Type of Compound

Authors investigating the buffering mechanism of the skin fail to mention that the type of epidermal constituents or amount of epidermal constituents available for binding/buffering an applied substance is dependent on the irritancy or corrosive nature of the test compound. For example, the more corrosive compounds will cause more epidermal degradation and cell destruction and hence make available more cellular membrane and intracellular constituents for binding and buffering. In addition, corrosive compounds will degrade the epidermal barrier and desmosomes of the keratinocytes leading to increased penetration.

In addition to the corrosive nature of the compound, the extent of its reactivity and its ability to trigger inflammation will determine what is available for binding/buffering. A compound that is more reactive has a greater chance of binding or being buffered. A compound that more easily triggers an inflammatory reaction may increase its penetration and the diversity of constituents for binding as the body recruits different components of our immune system to the inflamed site.

Young and How [73] devised an equation which will allow future researchers to assess the irritation potential of substances and preparations using the pH of the solution and the acid/alkali reserve (i.e., the buffer capacity). The problem with using the pH alone is that for some compounds the range of pH values between the concentration of a substance recognized as an irritant and that of the same substance recognized as a corrosive is narrow. This is recognized in the revision of the organisation for economic co-operation development (OECD) guideline 404 (acute dermal irritation/corrosion) [43]. Berner et al. provide data on pH, pK_a, irritation, and percutaneous penetration [3].

Note that none of the papers reviewed in this study first determined the irritant or corrosive nature of the applied compound/solution.

108.7 DISEASE SUSCEPTIBILITY

Individuals can be differentiated into three groups with high, medium, or low buffering capacity. Subjects with low buffering capacity are especially susceptible to the irritating effect of acids and bases, and predisposed to contact occupational eczema and dermatitis [20].

The common thread among these diseases is a diminished buffering capacity, an increased sensitivity to applied acids and bases, and in some cases an increased surface pH. Since we have established the importance of free AA to the neutralization ability of skin, it would make sense that a person with diminished buffering capacity would have either diminished free AA in total or AA of the certain types which function as buffering agents. If this is the case, the malfunction may be that of the common proteases on the skin which are responsible for releasing the free AA on the skin.

Goldsmith [19] notes many common proteases found in skin which are responsible for the free AA in the epidermis: these proteases such as serine, cysteine, aspartic, and metallo proteases work at pH optimisms of 7–9, 3–7, 2–7, and 7–9, respectively. Whether it is a shift in pH or a genetic defect, it seems likely that a defect in a protease on the epidermis will affect the composition and total amount of AA on the epidermis which will affect the buffering capacity and acidic/alkali sensitivity of an individual's skin.

108.8 CONCLUSION

Experimentation reviewed here suggests that AA are primarily responsible for the neutralization capacity of skin. The sources

as well as the types of AA that are primarily responsible for the neutralization capacity remain as speculation. In addition, it seems that a sweat component increases the neutralization capacity of the epidermis. Whether the buffering component of sweat is additional AA or lactic acid remains unknown.

Although additional components of the epidermis such as sebum, keratin, and CO_2 do not directly act as the primary buffering agents of the epidermis, they do play a role in the protection of skin from the harm caused by acids and bases. Sebum may slow down the initial penetration of applied substances. Keratin is important for the hydration of the skin which houses the water-soluble constituents responsible for buffering capacity and may contribute some of the free AA responsible for buffering-applied compounds. Finally, CO_2 may play a role in the buffering capacity of certain compounds or for long-term application.

When the buffering capacity is saturated and the pH of skin is shifted into an alkaline range, other endogenous mechanisms step in to restore the acidic skin pH [47]. Persons with diminished buffering capacity have an increased sensitivity to acids and bases, and may have an increased skin surface pH.

Although the existence of buffering system(s) is assured, further research needs to be completed to understand the mechanism, total capacity, and physiological source.

Taken together, we interpret this rich experimental literature as leading the way to utilization of contemporary methods to further refine our insight into the skin's buffering capacity. This capacity, when fully understood, may lead to the potential for not only decreasing toxicity of exogenous acids and bases but also establishing an experimental base for optimal pH in many pharmacologic, metabolic, and toxicologic situations.

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