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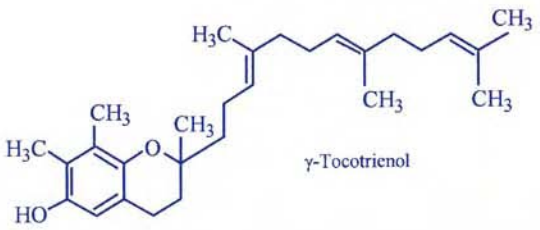

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Edited by Robert E. C. Wildman

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Handbook of Nutraceuticals and Functional Foods

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CRC Press
Boca Raton London New York Washington, D.C.

Library of Congress Cataloging-in-Publication Data

Handbook of nutraceuticals and functional foods / edited by Robert E.C. Wildman.
p. cm. (CRC series in modern nutrition)
Includes bibliographical references and index.
ISBN 0-8493-8734-5 (alk. paper)
1. Functional foods—Handbooks, manuals, etc. I. Wildman, Robert E. C., 1965- II.
Modern nutrition (Boca Raton, Fla.)

QP144.F85 H36 2000
613.2—dc21 00-057195
CIP

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International Standard Book Number 0-8493-8734-5
Library of Congress Card Number 00-057195
Printed in the United States of America 5 6 7 8 9 0
Printed on acid-free paper

Series Preface

The CRC Series in Modern Nutrition is dedicated to providing the widest possible coverage of topics in nutrition. Nutrition is an interdisciplinary, interprofessional field par excellence. It is noted by its broad range and diversity. We trust the titles and authorship in this series will reflect that range and diversity.

Published for a broad audience, the volumes in the CRC Series in Modern Nutrition are designed to explain, review, and explore present knowledge and recent trends, developments, and advances in nutrition. As such, they will appeal to professionals as well as to the educated layman. The format for the series will vary with the needs of the author and the topic, including, but not limited to, edited volumes, monographs, handbooks, and texts.

We welcome the contribution *Handbook of Nutraceuticals and Functional Foods*, edited by my young and talented colleague Robert E. C. Wildman. Dr. Wildman has assembled a stellar list of contributors on a topic of wide-ranging interest and application.

Ira Wolinsky, Ph.D.
University of Houston
Series Editor

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Preface

It may be difficult to imagine a more exciting time than today to be involved in nutrition research, education, and general health promotion. The investigative opportunities seem to be limitless and research tools range from large-scale epidemiology survey assessment to molecular biology and electron microscopy. Furthermore, scientific information can be shared rapidly and globally via a continuously growing number of journals, magazines, and Internet Web sites. The advent of many of the probing investigative techniques occurred in the latter half of the 20th century and has evolved to the current state of the art. These advances have allowed scientists to objectively investigate some of the most ancient concepts in the application of foods and the prevention and/or the treatment of diseases. As described in Chapter 1 our ancient ancestors recorded what they believed to be medicinal properties of certain foods. As the medical field developed and pharmaceutical companies flourished, many of these concepts were pushed aside and/or viewed as folklore. This was despite the fact that most of the early drugs and many today are actually derived from plants or are synthetic analogues of these substances.

For decades nutrition recommendations seemed to focus more upon "what not to eat" on a foundation consisting of the adequate provision of essential nutrients such as essential amino and fatty acids, vitamins, minerals, and water. Recommendations were to limit dietary substances such as saturated fatty acids, cholesterol, and sodium. Today scientists are recognizing that the other side of the nutrition coin, or "what to eat," may be just as important, if not more so. We have known for some time now that people who eat a diet rich in more natural foods, such as fruits, vegetables, nuts, whole grains, and fish, tend to lead a more disease-free life. The incidences of certain cancers and heart disease are noticeably lower than in populations that eat considerably lower amounts of these foods. For a while many nutritionists believed that this observation was more of an association rather than cause and effect. This is to say that the higher incidence of disease was more the result of higher meat consumption, body fat content, and lower activity levels associated with the lower consumption of fruits, vegetables, etc., rather than the lack of these foods. Thus, recommendations focused on limiting many of the "bad" food items by substituting them with foods that were not associated with the degenerative diseases, deemed "good" foods somewhat by default. With time and as research techniques advanced, scientists were able to better understand the composition of the "good" foods. Evidence quickly mounted to support earlier beliefs that many natural foods are seemingly prophylactic and medicinal.

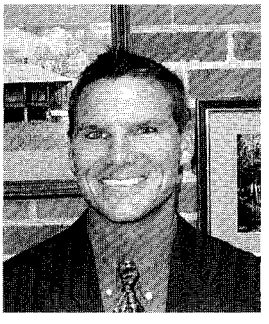
Today we find ourselves at what seems to be an epoch in understanding humanity's relationship with nature. Nutraceutical concepts remind us of our vast reliance upon other life forms on this planet. For it is these entities that not only provide us with our dietary essentials but also factors that yield protection against the environment in which we exist and the potentially pathological events we internally create. Food was an environmental tool used in the sculpting of the human genome. It is only logical to think then that eating more raw foods such as fruits and vegetables would lead to a healthier existence.

The advancement of scientific techniques has not only allowed us to better understand the diet we are supposed to eat, but it has also opened the door to one of the most interesting events in commerce. Food companies are now able to market foods with approved health claims touting the nutraceutical properties of the food. Food companies are also able to fortify existing foods with nutraceutical substances and/or create new foods designed to include one or more nutraceutical substances in their recipes. The opportunity afforded to food companies involved in functional foods appears without limitations at this time.

Despite the fact that this book reviews numerous nutraceuticals and functional foods, the field is still in its infancy. The best is probably yet to come. It is hard to imagine that nutrition science would ever be more exciting than this. But perhaps some scientist wrote that very same thought less than a century ago during the vitamin and mineral boom.

I truly hope you enjoy this book and welcome your comments and thoughts for future editions.

Editor



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Acknowledgments

The authors of the chapters of *Handbook of Nutraceuticals and Functional Foods* wish to acknowledge the following individuals and funding agencies for their support and assistance. Without the assistance of these individuals and resources, this project could not have been completed.

Richard Bruno and Dr. Robert Wildman would like to acknowledge the efforts of Dr. Steven Schwartz of the Department of Food Science and Nutrition at Ohio State University for his assistance in the development of Chapter 10.

Dr. Rosemary Wander would like to acknowledge the efforts of the following individuals for their assistance in the development of Chapter 19.

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Dr. Alfred H. Merrill and Dr. Eva Schmelz gratefully acknowledge the assistance of the numerous collaborators who have appeared as coauthors of their work. They wish to additionally acknowledge funding from the NCI (CA61820 and CA73327), Dairy Management, Inc., M & M Mars, the Georgia Research Alliance, and the Huguley Endowment.

Dr. Sidika Kasim-Karakas gratefully acknowledges the assistance of Maggi Emily Evans and Rogelio U. Almario.

Dr. Julie Mitchell would like to acknowledge the Ministry of Agriculture, Fisheries and Food (MAFF) and Scottish Office Agriculture Environment and Fisheries Department (SOAEFD) for their support.

Dr. Suzanne Hendrich and Dr. Patricia Murphy wish to acknowledge that their work was supported in part by the U.S. Army Medical Branch and Material Command under DAMD17-MM 4529EVM and by the Iowa Agricultural and Home Economic Experiments Station, Project 3375 and published as Journal Paper No. J-18633.

Dr. Robert Wildman would especially like to express his most sincere gratitude to those authors who contributed chapters to this book and his editor Lourdes Franco at CRC Press LLC. Dr. Wildman would also like to acknowledge the support of the College of Human Ecology at the University of Louisiana at Lafayette.

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1 Nutraceuticals: A Brief Review of Historical and Teleological Aspects

Robert E.C. Wildman

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I. INTRODUCTION

It only seems fitting that this book and others like it are published at the turn of the century and start of a new millennium. That these epochal points on the calender coincide with an explosion in interest and research into what has come to be the most exciting and promising areas of nutrition. *Nutraceuticals* as a nutritional/medical field is capturing the professional curiosity of nutritionists and health care professionals, as well as food scientists and the food industry. The growing financial commitment into research and education related to nutraceuticals as well as the development of professional seminars and conferences affirm that nutraceuticals will remain a cardinal priority as we make the transition to the 21st century and brave the new millennium.

In years gone by, at least in the United States, many aspects of nutraceuticals seemed to be placed under the umbrella of "alternative medicine." Even as little as a couple of decades ago, younger scientists were discouraged from pursuing a research direction founded upon topics such as flavonoids as the practical importance was generally criticized. However, today many of those areas listed under alternative medicine, such as herbal remedies and acupuncture, are now joining the ranks of conventional medicine therapies. Also many young researchers are indeed choosing to dedicate their research program to nutraceutical topics. Meanwhile other researchers, who are established in more traditional nutrition topics, are extending their research parameters to include nutraceutical investigations.

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TABLE 1.1
Definitions of Nutraceutical and Functional Foods

Nutraceuticals
Chemicals found as a natural component of foods or other ingestible forms that have been determined to be beneficial to the human body in preventing or treating one or more diseases or improving physiological performance. Essential nutrients can be considered nutraceuticals if they provide benefit beyond their essential role in normal growth or maintenance of the human body. An example is the antioxidant properties of vitamins C and E.
Functional Food
A food, either natural or formulated, which will enhance physiological performance or prevent or treat diseases and disorders. Functional foods include those items developed for health purposes as well as for physical performance. The Institute of Medicine's Food and Nutrition Board defined functional foods as "any food or food ingredient that may provide a health benefit beyond the traditional nutrients it contains."

As is true of any new area of investigation and discovery, scientists will struggle in the early years to develop and come to agreement on definitions as well as to coin terms and "buzz words." However, in this day and age of tremendous global interaction and availability of publishing resources the evolutionary process should not take as long as it might have a couple of decades ago. For the purposes of this book nutraceuticals will be considered components of traditional and exotic foods that have the potential to augment human health. The substance may (1) be part of an intact food source, such as the lycopene naturally occurring in tomato slice; or (2) be part of a processed food, such as lycopene from tomatoes in the recipe of catsup or salsa; or (3) be a fortified or enriched substance in a food, such as lycopene added to a fruit juice; or (4) be provided in supplemental form. Nutraceuticals are components in plants, animals, yeast, and fungi as well as bacteria. This is not to say that future functional foods will not include synthetic variants of natural nutraceuticals. If the substance is endowed to a plant it is often referred to as a *phytochemical.* Sometimes the term *medicinal botanicals* is used synonymously with phytochemicals. What has become obvious is that several "gray" or overlapping areas can develop between nutraceuticals, as dietary supplements, and pharmaceuticals, which then raises several regulatory issues. As many pharmaceuticals are actually substances extracted from plants this issue was inevitable and will require continued attention by regulatory bodies such as the Food and Drug Administration (FDA) in the United States.

II. GROWING AWARENESS OF NUTRACEUTICALS

At this time an increasing proportion of Americans and people around the world are more health conscious than ever before. Perhaps this is due to an increased awareness of what seem to be mostly preventable diseases (i.e., heart disease, cancers, osteoporosis, arthritis, and type 2 diabetes mellitus) coupled with an expansion in educational vehicles. Each year more and more newspaper and magazine articles are dedicated to the relationship between diet and health, and more specifically, to nutraceutical concepts. Furthermore, more health-related magazines and books grace the bookstore shelves than ever before. More television programs address topics of disease and prevention/treatment than ever before. But perhaps one of the most significant events to influence public awareness was the advent of the Internet (World Wide Web). The Internet provides a wealth of information regarding the etiology, prevention, and treatment of various diseases. Numerous Web pages have been developed by government agencies such as the U.S. Department of Agriculture (USDA; www.nal.usda.gov) and organizations such as the American Heart Association

(www.americanheart.org) and the American Cancer Society (www.cancer.org). Other information-based businesses such as CNN have information Web sites (i.e., www.WebMD.com) and Internet search engines exist to peruse medical abstracts (www.nlm.nih.gov/medlineplus).

Perhaps another reason for a greater collective health consciousness is a continuous shift in the population toward a more-advanced age. This is certainly the case in the United States. By extrapolating on current population trends scientists have estimated that by the year 2020 16% of the American population will be over the age of 65. And if population growth continues without impedance there will be 128 million more Americans in 2050 than there were in 1996, climbing from 265.2 to 393.9 million. Of this population; roughly 36% would be older than 50 years of age at the halfway point of the 21st century. Along with age comes an increased incidence of disease and thus individual focus upon prevention and treatment of such disorders.

Food companies are taking full advantage of the growing awareness of health. International companies such as Tropicana, General Mills, Central Soya, Abbott Laboratories have recognized a rapidly developing new market with tremendous promise and have dedicated hundreds of millions of dollars into the research of nutraceutical compounds, new product development, and marketing of these products (e.g., Health Claims). These products fall under a large category deemed *functional foods*. Functional foods are natural foods (i.e., fruits and vegetables) or manufactured food products that have bioactive compounds that can positively influence human function. For the purposes of this book, topics will be limited to functional foods containing nutraceutical compounds, meaning foods that may reduce the risk of disease or may possibly treat a disorder. However, functional foods can include foods specifically designed to improve performance (i.e., cognitive or physical). Thus products such as sport fluid and electrolyte replacements (i.e., Gatorade) and sport bars might be considered functional foods as well.

The functional food market has increased from $5.4 billion in 1992 to $8.9 billion in 1996. It is expected that this market will continue to grow steadily in the years to come. For instance, SoBe drinks, which contain ingredients such as creatine, choline, ginseng, ginko biloba, Echinacea, and vitamins and minerals, sold 1.1 million cases of its drinks in 1997. Over the next year its sales were reported to increase nearly fivefold. Other examples of functional foods include oatmeal-containing products and calcium- and folate-fortified orange juice. Benocol® is a margarine spread that uses soy components as recipe ingredients. It is specifically designed to help lower blood cholesterol levels, thus reducing the risk of heart disease. Furthermore, the Kellogg Company has developed a line of frozen entrees, breads, pasta, cookies, and other foods called Ensemble® that contain soluble fiber proven to lower blood cholesterol levels. Recently Ross Products, a division of Abbott Laboratories, has begun marketing Health Source™ Soy Protein Shakes. One shake contains roughly 20 g of soy protein. Their protein source is called Supro® XG, which is stated to be specially water processed to preserve the naturally high levels of soy isoflavones.

Perhaps one of the reasons nutraceuticals are so appealing to nutritionists and health care professionals is that the growing body of knowledge is supportive of the established general dietary recommendations for disease risk reduction made by government and private organizations. For instance, the 5-a-Day for Better Health program, which promotes the consumption of at least five servings of fruit and vegetables daily, is strongly reinforced by nutraceutical evidence. Five servings of fruits and vegetables is the minimum recommended intake of fruits and vegetables according to the Food Guide Pyramid (Figure 1.1). Survey work in the United States suggests that most of the population fails to eat this minimum recommended quantity. Other recommendations, such as those made by the American Dietetics Association (www.eatright.org), the American Heart Association and the American Cancer Society also encourage the consumption of fruits, vegetables, fish, and whole grain products.

Another important emerging concept in the field of nutraceuticals is that it is highly unlikely that there is a panacea or cure-all substance and that intact foods are probably more powerful than individual components. For instance, while β-carotene showed exceptional promise as a strong prophylactic compound according to epidemiological studies, when Finnish smokers were provided

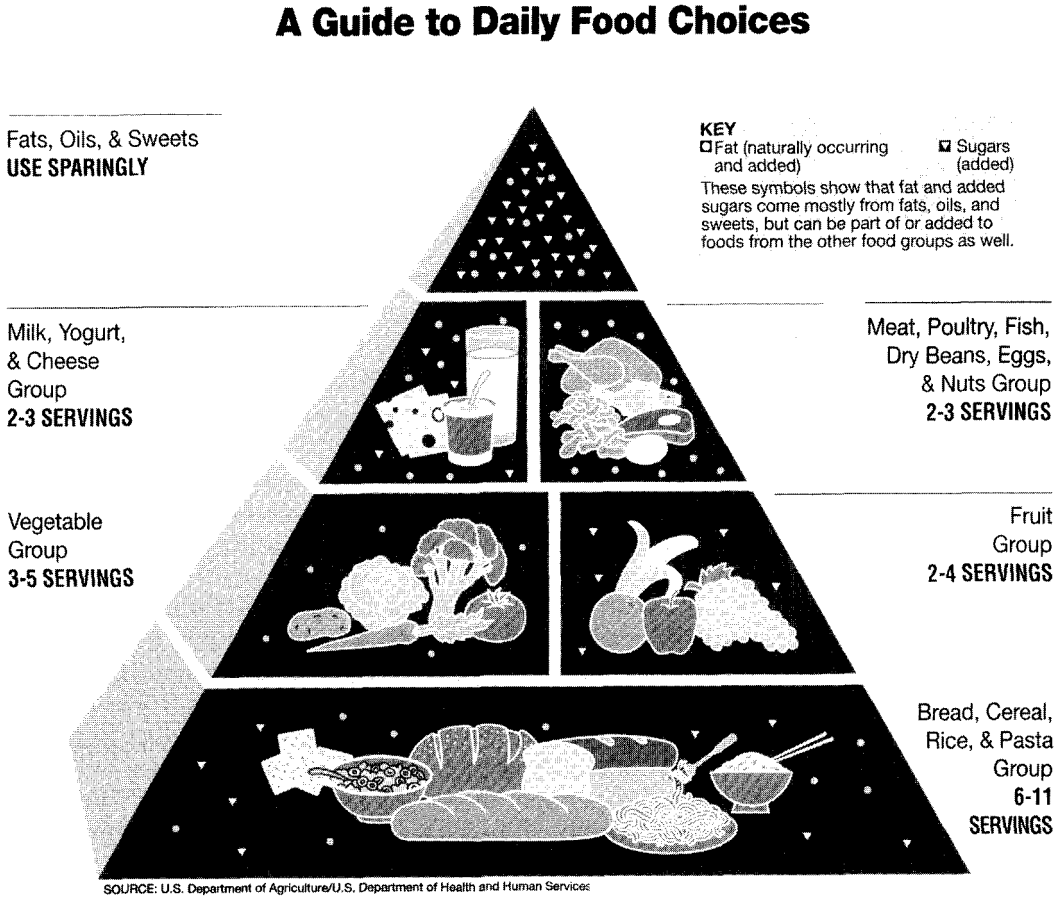


FIGURE 1.1 The Food Guide Pyramid.

supplements of β-carotene, the incidence of lung cancer actually increased.[1] The focus of nutraceutical consumption should be variety and balance. For instance, while the consumption of ω-3 polyunsaturated fatty acids can positively influence heart disease risk factors, overconsumption is presumed potentially disastrous. An important consideration for nutraceutical substances is that they are created by other life-forms with physiological intent as described below. Therefore, the potential for toxicity must always be considered. Most issues of toxicity for nutraceuticals and functional foods have not been adequately explored.

III. IN THE BEGINNING ...

It would seem from the statements above that the concept of nutraceuticals is a modern one. However, nothing could be farther from the truth. The notion that foods may possess the ability to prevent disease and/or be used as treatment of ailments dates back a couple millennia. Hippocrates proclaimed some 2500 years ago, "Let food be thy medicine and medicine be thy food." However,

the term *nutraceutical* and field of nutraceutical research are indeed modern. It really was not until the later decades of the 20th century that scientists were able to isolate food components precisely and also to perform proper clinical and laboratory investigations to test the efficacy of nutraceuticals to see if they did what our ancestors proclaimed they could do.

But how did our ancestors originally attain the notion that foods might have medicinal properties? The origin of functional foods is probably a combination of at least two things. First, our distant ancestors probably noticed that when animals where ailing they often ate certain plants that they would not otherwise eat. Second, unlike today where many aspects of our environment are fairly well explained, to our distant ancestors the activities of plants and other aspects of nature probably seemed magical.[2] For our ancestors living in regions of the world with changing seasons, each fall they observed the leaves on the tree change colors and eventually fall to the ground. In the meantime, the forests seemed to lose life, and the grass and flowers would wither and die as well. Then, as winter gave way to spring, the trees would bud and the leaves would reappear. Meanwhile, the grass would sprout from the ground as the flowers exploded in brilliant color. To our ancestors, these events must have seemed magical. Therefore, it probably did not take much convincing that the plants could provide medicinal properties as well. The fascination and medical and spiritual application of plants is recorded in the writings and artwork of ancient civilizations such as the Egyptians, Greeks, and Romans. Even Moses spoke to God, whose oracle was a burning bush. The mystical power of plants is sometimes also revealed in folklore. For instance, everyone knows that garlic is the bane of vampires. Meanwhile, the image of a woman laboring over a boiling cauldron, adding toadstools and herbs, such as mandrake and henbane, often comes to mind when one thinks of a witch.

Many past societies viewed sickness as punishment sent down from the gods. It is also legend that the treatment of ailments involved prayer to appease angry gods in conjunction with the consumption of "magic potions." These potions were developed via trial and error and mostly contained an herb or combinations of herbs. Early evidence of the medicinal application of plants include archaeological discoveries of a 60,000-year-old Neanderthal burial ground in present day Iraq.[3] In fact, many of these medicinal applications of plants are still popular in folk medicine today. Centuries later the Sumerians, who inhabited the region around the Tigris and Euphrates Rivers around 4000 B.C., scribed on clay tablets the medicinal use of licorice, opium, thyme, and mustard. It is also believed that the Babylonians who followed the Sumerians further developed the plant formulary to include senna leaves, saffron, coriander, cinnamon, and garlic.

The earliest medical textbooks were probably developed by the Egyptians. The *Ebers Papyrus* is perhaps the most famous of these works. It is named after the German Egyptologist George Ebers who purchased the works from an Arab in the latter part of the 19th century.[3] The Arab claimed to have come across the works in the necropolis just outside of the city of Thebes. It is believed that this work was originally developed in the 16th century B.C. and contains roughly 800 recipes and makes reference to over 700 drugs. Among the drugs are aloe, wormwood, peppermint, henbane, myrrh, mandragora, and hemp dogbane. The recipes instructed healers how to use these ingredients to create concoctions, wines, infusions as well as pills, salves, and poultices (plasters to be applied hot and wet). A treatment for diabetes mellitus is also said to be included in this text. A Chinese work entitled *Pen Tsao,* which dates back two millennia, is perhaps the earliest known Chinese pharmacopoeia.[2] This work was to be an authoritative up-to-date survey of the medicinal preparations of the time. Among its writings is a description of the application of a desert shrub called Chines ephedra (ma huang) to reduce fevers, improve circulation, suppress coughing, and relieve lung disorders. Even the writings of the Greek physician Hippocrates names some 300 to 400 healing plants. Hippocrates lived around 400 B.C. and is considered to be the Father of Modern Medicine.

The early writing of the medicinal application of plants may be viewed as the basis of modern pharmacological medicine. Even today more than half of the world's population still derives its medicine from the forests and fields. Also, many of the drugs commercially available today are either derived from plants or are synthesized to mimic plant compounds.

IV. TELEOLOGY OF NUTRACEUTICALS

One area of nutraceuticals that is often overlooked is the concept of *teleology*. As once stated by Dr. Robert DiSilvestro (author of Chapter 8) in a nutrition lecture to graduate students at the Ohio State University, "teleological is a big word like delicatessen." "It sounds impressive, however it has a very simple meaning. ... A delicatessen makes sandwiches and teleology means *why things are as the are*." Teleology is a concept that is most often untouched in nutrition lectures. Nutrition lectures usually provide a list of foods that contain a given nutrient but really do not explain what the physiological relevance of that substance to the organism that produced it. For instance, nutritionists will be quick to tell you that citrus fruits are a good source of ascorbic acid (vitamin C), yet very few would be able to tell you why. In past years this seemed okay. However, today it fails to address "the big picture" or holistic aspect of human nutrition. It also presents a very narrow perspective of human nutrition as it implies that other organisms on this planet are here to serve human needs. Along this line of logic then, plants make vitamin C to nourish people. The true purpose or teleology of the compound is lost. It is important to remember that from an evolutionary perspective, plants and most other organisms were here on this planet long before humans. And certainly bacteria were here before plants, which were here before animals. In fact, the presence of humans may be somewhat inconsequential to maintaining ecological balance as we exist at the zenith of the food chain and most foods we eat today are farmed.

Understanding the reasons nutraceutical compounds are present in different organisms may help us predict potential food sources of that substance as well as their impact on human function. There are numerous reasons for the production of nutraceutical substances by organisms. Often times, we overlook the fact that microbes, fungi, plants, and animals are organisms with the same general objectives as humans. They need to grow, mature, metabolize, defend themselves, and ultimately reproduce. Just as we will produce enzymes, hormones, antibodies, scar tissue, antioxidants, and so on specifically to serve our best interest, so will other organisms. For example, one purpose that carotenoids are produced by plants is to serve a role in photosynthesis and photoprotection. Plants also produce carotenoids as coloring pigments to attract animals such as insects and mammals for reproductive purposes. Some plants also produce interesting factors such as protease inhibitors that help them fend off insect and animal attack. It is important to remember that plants exist at the lower end of the food chain and thus are food for a full range of other organisms. As plants cannot run or fly away from a predator, they must stand their ground, so to speak. In doing so, they must create a host of interesting molecules to help defend themselves against microbes, mites, insects, and herbivores. Furthermore, as plants are stationary, reproduction of their species often depends upon luring insects and animals to them by producing coloring pigments and fragrances. This is especially true for plants that bear "fruit." Fruit is the mature product of a fertilized plant ovary or ovaries. The fruit unit consists of a seed or multiple seeds encased in a nutrient-dense environment with the energy mostly in the form of carbohydrate or oils. A seed is a fertilized and mature ovule, and is characteristically in the resting phase of the reproductive cycle. They will sprout given favorable environmental conditions. The energy of the fruit provides energy to the developing seed as well as nourishes an animal that consumes the fruit.

A. Primary and Secondary Metabolites in Plants

Plant cells produce many molecules that either do or do not seem to have direct relevance to the processes of growth and development. Those that do are often referred to as *primary metabolites* and include amino acids, chlorophyll, nucleotides, simple carbohydrates, and membrane lipids. Meanwhile *secondary metabolites* (secondary products or natural products) cannot be directly linked to processes such as photosynthesis, respiration, solute transport, translocation, and nutri-

ent assimilation.[4] For a while, scientists often regarded secondary metabolites as nonfunctional metabolites and waste products of plant metabolism. Also, although primary metabolites are ubiquitous throughout the plant kingdom, scientists recognized that specific secondary metabolites may only be associated with certain plant species or taxonomically related group. Scientists now recognize that many of these secondary metabolites are involved in defense operations that help protect a plant against herbivores and insects. Other secondary metabolites may be involved in plant-to-plant competition and/or attractants for pollinators and animals. Secondary metabolites can be divided into three groups: terpenes (isoprenoids), phenolic compounds, and nitrogen-containing secondary products such as alkaloids.

B. General Teleology of Select Nutraceuticals and Groups

What follows are some of the general teleological functions of a few of the more recognizable kinds of nutraceuticals. This overview is only meant to be an introduction and not an extensive review. One important concept stated earlier is reinforced in this section. Plants and other life-forms exist on this planet with the same basic objectives as humans, which certainly includes defense. Therefore, many nutraceutical substances may follow the same lines of toxicity as do pharmaceuticals. For example, plant alkaloids are produced as toxins to grazing or browsing animal. If the animal consumes a large enough quantity of the plant, a toxic effect is realized, according to substance threshold pharmacokinetics. In fact, each year large numbers of livestock deaths are related to the consumption of alkaloid-containing plants. This often happens when the animals are relocated to a new geography. What about humans? Is there the potential for similar toxic effects? Or, are the threshold ingestion levels for toxicity well above what humans ingest naturally? But what then happen when people experiment with supplements in self-medicating efforts. Thus, many questions concerning toxicity of nutraceuticals warrant investigation.

1. Carotenoids

Carotenoids are a broad category of plant molecules, which include the carotenes and xanthophylls, and are part of a larger class of molecules called terpenoids or isoprenoids (see Chapters 3 and 9). Carotenoids, as pigments, possess the ability to absorb visible light and appear colored. While their nutraceutical role to humans is mostly related to molecular protection against free radical attack, carotenoids are produced by plants as photosynthetic pigments and as photoprotective entities. This is to say that carotenoids, along with the two other kinds of photosynthetic pigments (chlorophylls and phycobilins) are involved in the harnessing of light energy into carbohydrate structures. Having several types of pigments (with subtypes) allows a greater wavelength range of light absorption. The pigments are attached to proteins in photosynthetic structures in plant chloroplast membranes. Carotenoids are produced by plants for reproductive purposes as well. For instance, the colorization of seed-bearing fruits will attract animals while the colorization of flowers can serve to attract and guide insects.

The carotenoids are involved in photosynthesis in two ways. First, carotenoids can function as light-harvesting structures that will then pass the energy to chlorophyll. β-Carotene is commonly found as part of the light-harvesting apparatus of plants; however, this activity may be more of a general role of the xanthophylls than the carotenes. The greater role of the carotenes may be viewed as more protective in nature as the carotenes appear to serve as a "sink" for triplet states created during the excitation of chlorophyll by light. The triplet state is more reactive with other molecules such as molecular oxygen (O_2). This would create reactive oxygen species (free radicals) that would be detrimental to biological membranes and other molecules in plant tissue.

In fruits and vegetables chloroplasts differentiate into organelles called chromoplasts. In the process they lose the ability to produce chlorophyll and synthesize a variety of yellow, red, or orange carotenoid pigments. For example, as a tomato ripens and chloroplasts change into chro-

moplasts, less and less chlorophyll and photosynthetic enzymes will be produced while more and more of the red carotenoid pigment lycopene is synthesized (see Chapter 10). One important result of this activity might be to change the aesthetic characteristics of the seed-bearing fruit. For instance, it is important for the reproduction of the tomato plant for its seed-bearing fruit to be consumed by an animal. As the animal moves away from the original plant, it will eventually defecate the seeds in another location, hopefully fertile ground.

2. Conjugated Linoleic Acid

Conjugated linoleic acid (CLA) is found primarily in beef and milk. As discussed by Watkins in Chapter 27, CLA is mainly 18:2 ω-9(cis), 11(trans) and 18:2 ω-10(trans), and 12(cis). Experimental evidence suggests that CLA has anticarcinogenic properties, can slow the progression of atherosclerosis, and can stimulate key immune system events. Other evidence suggests that CLA may inhibit lipogenesis while also increasing some mechanisms involved in fatty acid use (i.e., CPT). Because of its food source, one might conclude that CLA is produced by cows. However, CLA is produced by specific bacteria in the rumen via modification of linoleic acid in the animal's diet. It is then absorbed by the ruminant and enters its tissue, including mammary tissue and skeletal muscle (beef).

At this point there are several possible explanations for microbial production of CLA. First, it could be that CLA is simply an excretory metabolite of bacteria or even a metabolic intermediate that has been released by the bacteria. While this certainly is possible, it hardly seems plausible. Second, CLA could be produced for symbiotic purposes, thus promoting the health and longevity of the cow, which is serving as the host organism for the bacteria. Last, CLA may be produced by rumen bacteria as a factor specific to promoting the preservation of that bacteria species in rumen. Here CLA may act by influencing the environment, by controlling the proliferation of competitive bacteria, or may serve as a growth factor for its own population. Some evidence for the role(s) of CLA may be derived from other unique fatty acids associated with the rumen. It is known that the bacteria that digest cellulose (cellulolytic) require or are at least stimulated by branch chain fatty acids (BCFA). Also, BCFA may actually increase the milk yield of a cow, thus helping improve the health and longevity of the host organism.

3. Flavonoids

Flavonoids are a broad category of compounds produced by plants and many appear to have nutraceutical potential by lowering blood cholesterol levels, osteoporotic and carcinogenic events, as well as perhaps enhancing antioxidant capabilities (see Chapters 4 through 8, 14, and 15). Flavonoids are produced and used by plants in a variety of ways. One interesting role of flavonoids is their involvement in symbiotic nitrogen fixation. All plants require a source of reduced nitrogen. Despite the fact that the most abundant atmospheric gas is nitrogen (N_2), plants are unable to use this nitrogen directly. Thus, plants must rely upon reduced nitrogen in the soil or a symbiotic presence of nitrogen-reducing (fixing) bacteria. The reduced form of N_2 is ammonia (NH_4^+). For the most part, symbiotic nitrogen-fixing bacteria are known as Rhizobiaceae. These are soil-dwelling bacteria that mostly belong to the genus *Rhizobium*. The symbiotic relationship is somewhat limited to members belonging to a plant family known as Leguminosae, although other relationships exist.[7] Among the members of the family are soybeans, peas, and beans. Nitrogen fixation in *Rhizobium* bacteria occurs via a nitrogenase reaction and only takes place after the symbiotic relationship has been established. This requires that the bacteria enter the plant root and differentiate into bacteroid organisms that may be as much as 40-fold larger and club shaped.[8] As it seems, the plant roots exude flavonoid substances such as luteolin, hesperitin, and diadzein which induce or repress key genes (*nod*) in the bacteria. It would seem that these factors bind with a nod protein typically expressed. Once the interaction has occurred, the newly

formed complex then binds with the promoter region of *nod* genes resulting in the induction or repression of the expression of proteins which would not occur in the absence of these flavonoids. These proteins promote the activities involved with the formation of the nitrogen-fixing nodules which contain groups of differentiated bacteroids in the hairs of plant roots. In the absence of flavonoids nitrogen fixation is absent.

The incorporation of bacteria into root structures should not be viewed as bacterial infection as it is a healthy and necessary con-habitation. Conversely, plant flavonoids will also work to inhibit undesirable bacterial infection. In this role they function as antibiotics (phytoalexins) which perhaps in a similar manner inhibit the growth of invading bacteria.

Anthocyanins are pigmented flavonoids that are responsible for red, pink, purple, and blue colors. While these flavonoids have nutraceutical potential for humans, their true purpose is to attract animals for seed and pollen dispersal. Other flavonoids can absorb light at wavelengths shorter than anthocyanins and thus cannot be seen with the naked human eye. However, bees and other insects that can see farther into the ultraviolet (UV) range of the spectrum can be attracted.[5] While this function is more related to flowers, some of the same and similar flavonoids are found in leaves of green plants to absorb potentially harmful UV-B radiation (280 to 320 nm) while allowing the photosynthetically active light to pass uninterrupted.

4. Nitrogen- and Sulfur-Containing Amino Acid Derivatives

Plants produce a host of secondary metabolites that contain nitrogen. Among these structures are alkaloids and cyanogenic glycosides which are derivatives of common amino acids. Alkaloids appear in roughly 20% of the species of vascular plants.[5] These substances, such as the highly recognizable cocaine, nicotine, morphine, and caffeine, are known to have striking physiological effects in vertebrates. Alkaloids were once believed to be a vehicle for nitrogenous waste, somewhat like urea and uric acid in animals. They were also believed to be nitrogen storage molecules. However, scientists now believe alkaloids to be defense molecules against predators, especially mammals, due to their general toxicity.[5]

Capsaicinoids (see Chapter 13) are alkaloid structures produced by pepper fruits and are derived from the amino acids phenylalanine and valine or leucine as well as branch chain fatty acids. Capsaicin is used medicinally to treat many medical conditions, including arthritis, and may have anticarcinogenic and antioxidant qualities. Capsaicinoids can irritate the dermal surface of animals and evoke a "hot" sensation when consumed via interaction with pain receptors. This can deter animals from consuming the fruit. Meanwhile cruciferous vegetables (see Chapter 11) contain glucosinolates that are by themselves inert, but can be converted to toxic metabolites when plant tissue is traumatized and the contents of different plant tissue mix. These substances are responsible for the smell associated with some cruciferous vegetables such as cabbage, broccoli, and radishes, which may be a deterrent to animals. The derived substances include isothiocyanate, thiocyanate, and nitrile and are created when plant tissue trauma allows glucosinolates from certain cells to mix with enzymes in other cells.

5. Proteinase and α-Amylase Inhibitors

Among the defensive arsenal of some plants are protease or proteinase inhibitors. Bowman Birk Inhibitor (BBI) is one such substance in soy and is believed to have anticarcinogenic properties.[9] Protease inhibitors are a defense mechanism for plants. For instance, tomatoes, legumes, and other plant components will produce these substances in an attempt to hinder the protein-digesting enzyme activity of herbivores and insects.[5] As plant tissue enters the gut of a herbivore, the protease inhibitors bind to proteases such as trypsin and chymotrypsin and reduce their activity. This is also true for insects. In addition to protease inhibitors, plant tissue also produce α-amylase inhibitors that inhibit the activity of the starch digesting α-amylase. Plants also produce substances called

lectins. These substances bind to carbohydrates and carbohydrate-containing proteins. Lectins then interfere with the absorption of nutrients in the gut of the animal feeding on the plant. While protease inhibitors and other digestive process inhibitors may seem like an "after the fact" mechanism, their true significance is probably related to the future and not the present. Insects and herbivores that chronically feed on these plants may not be properly nourished and may choose their future meals elsewhere. Or, a chronic consumer may become ill and die and/or fail to reproduce future consumers of the plant.

6. Omega-3 PUFA

The nutraceutical classification of ω-3 PUFA is based upon their inverse relationship to heart disease (see Chapters 18 and 20) and probably certain cancers and inflammatory disorders such as arthritis (see Chapter 22). Humans ingest ω-3 PUFA primarily in plant and fish oils, which suggests that both plants and animals can make these PUFA. However, animals lack the desaturating enzymes necessary to create ω-3 PUFA; thus the ω-3 PUFA found in fish and other marine animals are derived from their diet. Plants create ω-3 PUFA primarily to serve as components of glyceroglycolipids and glycerophospholipids (phospholipids). These molecules are polar lipids and serve as the main structural lipids in plant membranes.[10] Several of these molecules are conceptually the same as phospholipids in animals, including phosphotidylinositol, phosphatidylethanolamine, and phosphotidylethanolamine. In addition, carbohydrate moieties can replace phosphate to create monogalactosyldiacylglycerol (MGD) and digalactosyldiacylglycerol (DGDG).

There are at least six different fatty acids employed by plants to make structures such as the ones mentioned above. These include palmitic acid (16:0), linoleic acid (18:2 ω-6), and linolenic acid (18:3 ω-3). The nature of the lipid composition has been suggested to be involved, but not to be the primary determinant in plant sensitivity to chilling temperatures. Also, another role of linolenic acid in plant membranes is perhaps far more fascinating. When plant cells are wounded by animal or insect feeding, cells in the traumatized tissue, such as the leaf, release systemin. Systemin is an 18-amino acid plant hormone, perhaps the only plant hormone. This hormone is circulated in the plant phloem and binds to a receptor site on other cells. This results in the biosynthesis of jasmonic acid (Figure 1.2). Jasmonic acid is produced from linolenic acid (18:3 ω-3 PUFA) stored in the plasma membrane. What is most intriguing is how this activity is strikingly similar to eicosanoid production in mammalian cells. Jasmonic acid will then induce the expression of protease inhibitors.

Algae and phytoplankton is a major food for many fish. The majority of the derived fatty acids is oleic acid, linoleic acid, and linolenic acids. All of these fatty acids can be elongated and further desaturated by fish. For example, linolenic acid can be elongated and desaturated to form the prevalent ω-3 PUFA in fish lipids: DHA (docosahexaenoic acid, 22:6 ω-3) and EPA (eicosapentaenoic acid, 20:5 ω-3). For example, Morris and Schneider[11] reported back in 1969 that the brain fatty acids of Antarctic fish were particularly dense in 24-carbon ω-3 PUFA. In comparison with fish that swim closer to the surface (temperate-water), deep-water fish appear to possess higher proportions of PUFA (42 vs. 33%) and a lower proportion of SFA (17 vs. 35%).[12,13] Therefore, the longer PUFA can to some degree offset the crystallizing effect of lower environmental temperatures as well as the physical influence of higher environmental pressure.[13] Furthermore, the ω-3: ω-6 ratio of cell membranes is higher in marine life then in land mammals. For instance, it has been reported that catfish mitochondrial membranes present a ratio of 3.85 whereas a rat presents a ratio of 0.01.[14] Like fish, crustaceans (lobster, crab, shrimp) also require unsaturated fatty acids in their diet.[15] Likewise, their tissue will present PUFA in amounts that do reflect their diet. The fatty acid composition of the adult brine shrimp will show about 14 to 15% ω-3 PUFA and about 6 to 7% ω-6 PUFA.

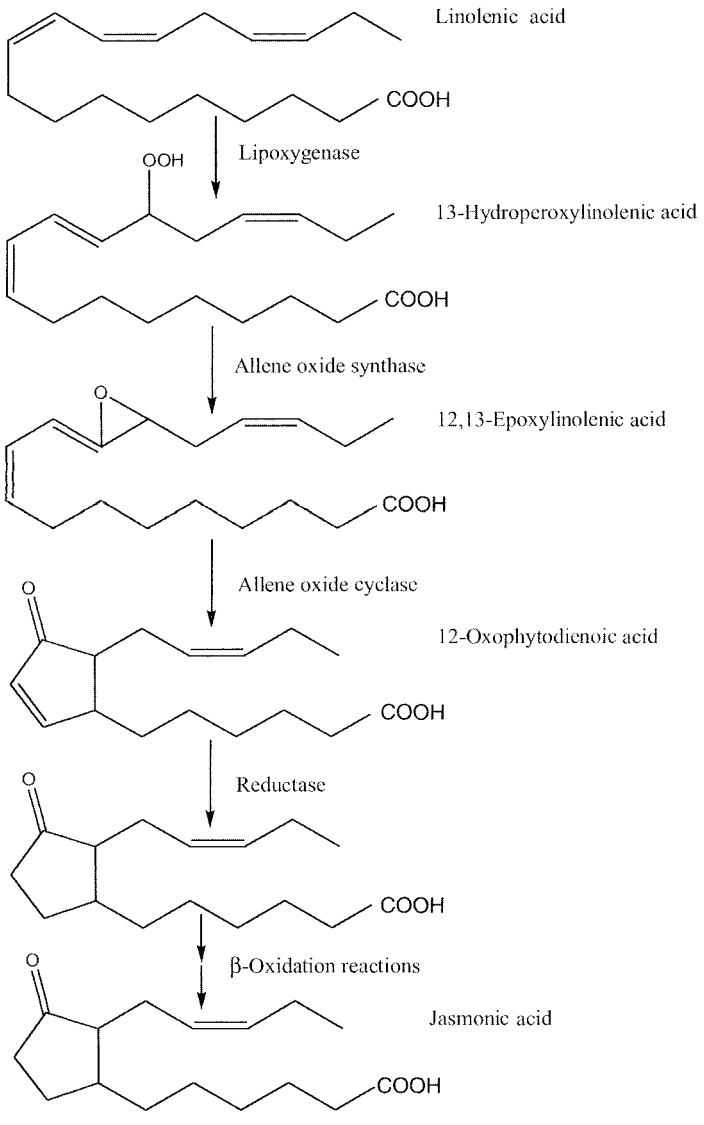
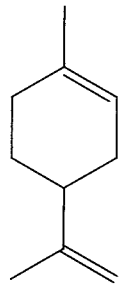


FIGURE 1.2 Conversion pathway for the formation of jasmonic acid from linolenic acid.

7. Terpenoids

Many of the monoterpenes and their derivatives are toxic agents to insects. In fact, some of these monoterpenes, such as the monoterpene esters called pyrethroids, are actually used as components of commercial insecticides. Most plants contain so-called *essential oils*, which are a mixture of volatile monoterpenes and sesquiterpenes. These oils have insect-repellent properties and are found in glandualar hairs protruding from the epidermis or in the peel of fruits. These serve to protect the plant by advertising the potential toxicity of the plant. The highly touted nutraceutical compound limonene (Figure 1.3) is found in the essential oils of citrus peels. Meanwhile menthol is the chief monoterpene in peppermint essential oil. In an interesting twist, some plants release certain monoterpenes and sesquiterpenes only after insect feeding has already begun. While these terpenoids may not be necessarily toxic to the feeding insect, they do serve to attract predator insects that



d-Limonene

FIGURE 1.3 Structure of *d*-limonene.

feed on the feeding insects. In addition, numerous diterpenes have been shown to be toxins and skin irritants to deter herbivores and insects.

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2 Classifying Nutraceuticals

Robert E.C. Wildman

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I. INTRODUCTION

As described in Chapter 1, the application of foods to prevent or treat certain ailments is chronicled in ancient drawings and writings. However, large-scale laboratory and clinical investigation to identify the active substances (nutraceuticals) and to challenge their efficacy has really only occurred over the last few decades of the 20th century. Among the nutraceutical emissaries, at least in the Western World, were plant fibers and β-carotene and ω-3 polyunsaturated fatty acids (PUFA). Today the number of purported nutraceutical substances is in the hundreds and some of the more popular substances include isoflavones, tocotrienols, allyl sulfur compounds, conjugated linoleic acid (CLA), and carotenoids. Not only have nutraceuticals captured the interest of scientists and health care practitioners as evidenced by an explosion of scientific articles, but nutraceutical concepts are becoming very mainstream as well. In light of a long and growing list of nutraceutical substances and the functional foods that serve as their vehicle of consumption, organization systems have become warranted to allow for greater comprehension and application.

Depending upon one's interest and/or background, the appropriate organizational scheme for nutraceuticals can vary. For example, cardiologists may be most interested in those nutraceutical substances that are associated with reducing the risk factors of heart disease. Specifically their interest may lie in substances purported to positively influence hypertension and hypercholesterolemia and to reduce free radical activity. Meanwhile, oncologists may be more interested in those substances that are more associated with anticarcinogenic activities. These substances may be associated with augmentations of microsomal detoxification systems and antioxidation defenses, or they may slow the progression of existing cancer. Thus, their interest may lie in

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both chemoprevention or potential adjunctive therapy. On the other hand, the nutraceutical interest of food scientists working on the development of a functional food product will not only include physiological properties, but also stability and sensory properties, as well as issues of cost efficiency. For example, the anticarcinogenic triterpene limonin is lipid soluble and intensely bitter, somewhat limiting its commercial use as a functional food ingredient.[1] However, the glucoside derivative of limonin, which shares some of the anticarcinogenic activity of limonin, is water soluble and virtually tasteless thereby enhancing its potential use as an ingredient.[2]

Health promotion specialists may be more concerned about the nutraceutical potential of intact foods and how to apply those foods in practical dietary recommendations. Meanwhile, individuals adhering to a specific philosophical or medical diet may be concerned about the origin of a nutraceutical substance and the possibilities of deficiencies, as well as toxicity, in their diet. For example, a strict vegetarian (vegan) would certainly consume a diet relatively rich in flavonoids, carotenoids, and tocotrienols; however, their diet would be relatively deficient in CLA as well as possibly provide a less-than-favorable ω-6:ω-3 PUFA ratio. Last, the interest in an organizational model for nutraceuticals may be for instructional purposes for undergraduate and graduate and professional students (i.e., food science, nutrition, nursing, medicine, and pharmacy).

II. ORGANIZATIONAL MODELS FOR NUTRACEUTICALS

Whether it be for academic instruction, clinical trial design, functional food development, or dietary recommendations, nutraceuticals can be organized in several ways depending upon the specific interest or need at hand. This chapter briefly describes ways of organizing nutraceuticals based upon:

- Food source
- Mechanism of action
- Chemical nature

III. FOOD SOURCE

A. Animal, Plant, Microbial

One of the broader models of organization for nutraceuticals is based upon their potential as a food source to humans. Here nutraceuticals may be separated into plant, animal, and microbial (i.e., bacteria and yeast) groups. For example, Dr. Clare Hasler presented some of the major functional foods and their inherent nutraceuticals based upon animal and plant sources in a review published in *Food Technology*.[3] Grouping nutraceuticals as provided by plants, animals, or microbes holds numerous merits and can be a valuable tool for diet planning as well classroom and seminar instruction. However, one interesting consideration with this organization system is that the food source may not necessarily be the organism of origin for one or more substances. An obvious example is CLA which is part of the human diet mostly as a component of beef and dairy foods. However, it is actually made by bacteria in the rumen of the cow. Therefore, issues involving the food chain or symbiotic relationships may be a consideration for some individuals working with this organization scheme. Also, because of fairly conserved biochemical aspects across species, many nutraceutical substances are found in both plants and animals, as well as at times in microbes. For example, microbes, plants, and animals contain choline and phosphotidylcholine. This is also true for sphingolipids; however, plants and animals are better sources. Also, linolenic acid (18:3 ω-3 PUFA) can be found in a variety of food resources including animal flesh despite the fact that it is primarily synthesized in plants and other lower members of the food chain. Table 2.1 presents some of the more recognizable nutraceutical substances grouped according to food source providers.

TABLE 2.1
Examples of Nutraceutical Substances Grouped by Food Source

Plants		Animal	Microbial
β-Glucan	Allicin	Conjugated Linoleic Acid	*Saccharomyces boulardii*
Ascorbic acid	*d*-Limonene	(CLA)	(yeast)
γ-Tocotrienol	Genestein	Eicosapentaenoic acid (EPA)	*Bifidobacterium bifidum*
Quercetin	Lycopene	Docosahexenoic acid (DHA)	*B. longum*
Luteolin	Hemicellulose	Spingolipids	*B. infantis*
Cellulose	Lignin	Choline	*Lactobacillus acidophilus*
Lutein	Capsaicin	Lecithin	(LC1)
Gallic acid	Geraniol	Calcium	*L. acidophilus* (NCFB 1748)
Perillyl alcohol	β-Ionone	Ubiquinone (coenzyme Q_{10})	*Streptococcus salvarius*
Indole-3-carbonol	α-Tocopherol	Selenium	(subs. Thermophilus)
Pectin	β-Carotene	Zinc	
Daidzein	Nordihydrocapsaicin		
Glutathione	Selenium		
Potassium	Zeaxanthin		

Note: The substances listed in this table include those that are either accepted or purported nutraceutical substances.

B. Nutraceuticals in Specific Foods

In an organization model related to the one above, nutraceuticals can be grouped based upon relatively concentrated foods. This model is more appropriate when there is interest in a particular nutraceutical compound or related compounds or when there is interest in a specific food for agricultural/geographic reasons or functional food development purposes. For example, the interest may be in the nutraceutical qualities of a local crop or a traditionally consumed food in a geographic region, such as pepper fruits in the southwestern United States, olive oil in Mediterranian regions, and red wine in western Europe and Northern California. There are several nutraceutical substances that are found in higher concentrations in specific foods or food families. These include capsaicinoids which are found primarily in pepper fruit and allyl sulfur (organosulfur) compounds which are particularly concentrated in onions and garlic. Table 2.2 provides a listing of certain nutraceuticals that are considered unique to certain foods or food families. One consideration for this model is that for several substances, such as those just named, there is a relatively short list of foods that are concentrated sources. However, the list of food sources for other nutraceutical substances can be much longer and can include numerous seemingly unrelated foods. For instance, citrus fruit contain the isoflavone quercetin, as do onions, a plant food of seemingly little relation. Citrus fruit grow on trees while the edible bulb of the onion plant (an herb) develops at ground level. Other plant foods with higher quercetin content are red grapes; but not white grapes, broccoli, which is a cruciferous vegetable; and the Italian yellow squash. Again, these foods appear to bear very little resemblance to citrus fruit, or onions for that matter. On the other hand, there are no guarantees that closely related or seemingly similar foods contain the same nutraceutical compounds. For example, both the onion plant and the garlic plant are perennial herbs arising from a rooted bulb, and are also cousins in the lily family. However, although onions are loaded with quercetin, with some varieties containing up to 10% of their dry weight of this flavonoid, garlic is quercetin void. Resources are available on the Internet to assist individuals sort this out (http://www.ars-grin.gov/duke).

TABLE 2.2
Examples of Foods That Have Higher Content of Specific Nutraceutical Substances

Nutraceutical Substance/ Family	Foods of Remarkably High Content
Allyl sulfur compounds	Onions, garlic
Isoflavones	Soybeans and other legumes, apios
Quercetin	Onion, red grapes, citrus fruit, broccoli, Italian yellow squash
Capsaicinoids	Pepper fruit
EPA and DHA	Fish oils
Lycopene	Tomatoes and tomato products
Isothiocyanates	Cruciferous vegetables
β-Glucan	Oat bran
CLA	Beef and dairy
Resveratrol	Grapes (skin), red wine
β-Carotene	Citrus fruit, carrots, squash, pumpkin
Carnosol	Rosemary
Catechins	Teas, berries
Adenosine	Garlic, onion
Indoles	Cabbage, broccoli, cauliflower, kale, Brussel sprouts
Curcumin	Tumeric
Ellagic acid	Grapes, strawberries, raspberries, walnuts
Anthocyanates	Red wine
3-*n*-Butyl phthalide	Celery
Cellulose	Most plants (component of cell walls)

Note: The substances listed in this table include those that are either accepted or purported nutraceutical substances.

IV. MECHANISM OF ACTION

Another means of classifying nutraceuticals is by their mechanism of action. This system groups nutraceuticals together, regardless of food source, based upon their proven or purported physiological properties. Among the classes would be antioxidation, antibacterial, hypotensive, hypocholesterolemic, antiaggregate, anti-inflammatory, anticarcinogenic, osteoprotective, and so on. Similar to the scheme just discussed, credible Internet resources may prove invaluable to this approach. Examples are presented on Table 2.3. This model would also be helpful to an individual who is genetically predisposed to a particular medical condition or to scientists trying to develop powerful functional foods for just such a person. The information in this model would then be helpful in diet planning in conjunction with the organization scheme just discussed and presented in Table 2.2. However, as eluded to numerous times in this book, many issues related to toxicity, synergism, and competition associated with nutraceuticals and their foods are not yet known.

What may be of interest is that there are several nutraceuticals that can be listed as having more than one mechanism of action. One of the seemingly most versatile nutraceutical families is the ω-3 PUFAs. Their nutraceutical properties can be related to direct effects as well as to some indirect effects. For example, these fatty acids are used as precursors for eicosanoid substances that locally vasodilate, bronchodilate, and deter platelet aggregation and clot formation. These roles can be prophylactic for asthma and heart disease. Omega-3 PUFA may also reduce the activities of protein kinase C and tyrosine kinase, both of which are involved in a cell growth signaling mechanism. Here, the direct effects of these fatty acids may reduce cardiac hypertrophy and cancer cell proliferation. Omega-3 PUFA also appear to inhibit the synthesis of fatty acid synthase (FAS) which is a principal enzyme complex involved in de novo fatty acid synthesis. Here the nutraceutical

TABLE 2.3
Examples of Nutraceuticals Grouped by Mechanisms of Action

Anticancer	Positive Influence on Blood Lipid Profile	Antioxidation	Anti-inflammatory	Osteogenetic or Bone Protective
Capsaicin	β-Glucan	CLA	Linolenic acid	CLA
Genestein	γ-Tocotrienol	Ascorbic acid	EPA	Soy protein
Daidzein	δ-Tocotrienol	β-Carotene	DHA	Genestein
α-Tocotrienol	MUFA	Polyphenolics	Capsaicin	Daidzein
γ-Tocotrienol	Quercetin	Tocopherols	Quercetin	Calcium
CLA	ω-3 PUFAs	Tocotrienols	Curcumin	
Lactobacillus acidophilus	Resveratrol	Indole-3-carbonol		
Sphingolipids	Tannins	α-Tocopherol		
Limonene	β-Sitosterol	Ellagic acid		
Diallyl sulfide	Saponins	Lycopene		
Ajoene		Lutein		
α-Tocopherol		Glutathione		
Enterolactone		Hydroxytyrosol		
Glycyrrhizin		Luteolin		
Equol		Oleuropein		
Curcumin		Catechins		
Ellagic acid		Gingerol		
Lutein		Chlorogenic acid		
Carnosol		Tannins		
L. bulgaricus				

Note: The substances listed in this table include those that are either accepted or purported nutraceutical substances.

effect may be considered indirect, as chronic consumption of these PUFA may theoretically lead to lesser quantities of body fat over time and the development of obesity. The obesity might then lead to the development of hyperinsulinemia and related physiological aberrations such as hypertension and hyperlipidemia.

V. CHEMICAL NATURE

Another method of grouping nutraceuticals is based upon their chemical nature. This approach allows nutraceuticals to be categorized under molecular/elemental groups. This preliminary model includes several large groups which then provide a basis for subclassification or subgroups, and so on. One way to group nutraceuticals grossly is as follows:

- Isoprenoid derivatives
- Phenolic substances
- Fatty acids and structural lipids
- Carbohydrates and derivatives
- Amino acid-based substances
- Microbes
- Minerals

As scientific investigation continues, several hundred substances will probably be deemed nutraceuticals. As many of these nutraceutical compounds appear to be related in synthetic origins or molecular nature, there is the potential to broadly group many of the substances together

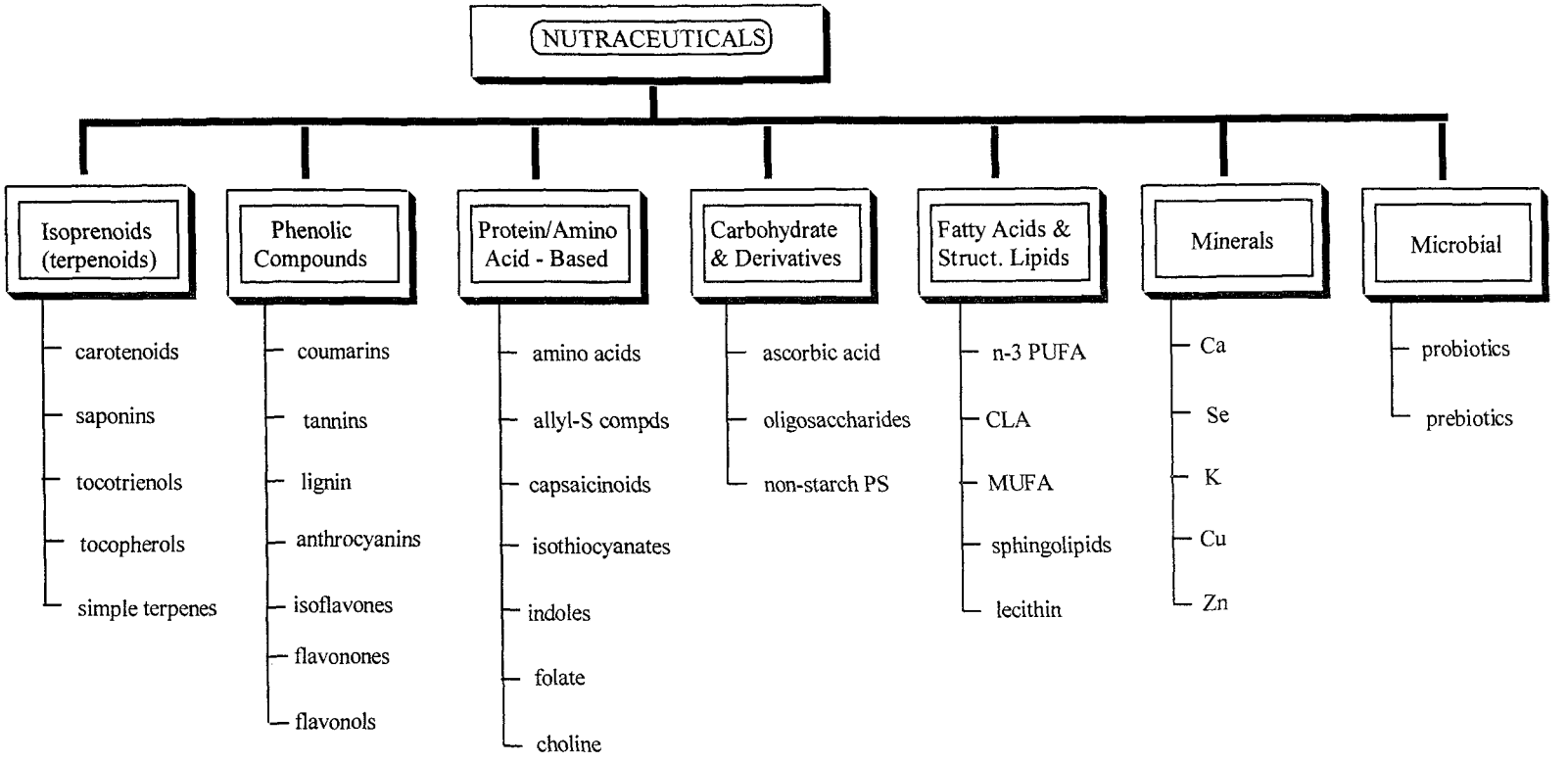
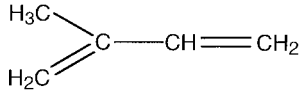


FIGURE 2.1 Organizational scheme for nutraceuticals.

(Figure 2.1). This scheme is by no means perfect and it is offered in "pencil," not "etched in stone." It is expected that scientists will ponder this organization system, find flaws, and suggest ways to evolve the scheme, or disregard it completely in favor of a better direction. Even at this point several "gray" areas are apparent. For instance, mixtures of different classes can exist, such as mixed isoprenoids, prenylated coumarins, and flavonoids as discussed in Chapter 3. Also, phenolic compounds could arguably be grouped under a very large "Amino Acid and Derivatives" category. Although most phenolic molecules arise from phenylalanine as part of the shikimic acid metabolic pathway, other phenolic compounds are formed via the malonic acid pathway, thereby circumventing phenylalanine as an intermediate. Thus, phenolics stand alone as their own group whose most salient characteristic is chemical structure, not necessarily synthetic pathway.

A. Isoprenoid Derivatives (Terpenoids)

Isoprenoids and terpenoids are terms used to refer to the same class of molecules. These substances are without question one the largest groups of secondary metabolites (see Chapter 1). In accordance, they are also the basis of many plant-derived nutraceuticals. Under this large umbrella are many popular nutraceutical families such as carotenoids, tocopherols, tocotrienols, and saponins. This group is also referred to as isoprenoid derivatives because the principal building block molecule is isoprene (Figure 2.2). Isoprene itself is synthesized from acetyl coenzyme A (CoA) in the well-researched mevalonic acid pathway (Figure 2.3) and glycolysis-associated molecules pyruvate and 3-phosphoglycerate in a lesser-understood metabolic pathway.[4] In both pathways the end product is isopentenyl phosphate (IPP) and IPP is often regarded as the pivotal molecule in the formation of larger isoprenoid structures. Once IPP is formed, it can reversible isomerize to dimethyallyl pyrophosphate (DMAPP) as presented in Figure 2.4. Both of these five-carbon structures are then used to form geranyl pyrophosphate (GPP) which can give rise to monoterpenes. Among the monoterpenes are the highly touted *d*-limonene and perillyl alcohol. Both of these monoterpenes are discussed by Crowell and Elson in Chapter 3.



Isoprene

FIGURE 2.2 Isoprene.

GPP can also react with IPP to form the 15-carbon structure farnesyl pyrophosphate (FPP) which then can give rise to the sesquiterpenes. FPP can react with IPP or another FPP to produce either the 20-carbon geranylgeranyl pyrophosphate (GGPP) or the 30-carbon squalene molecule, respectively. GGPP can give rise to diterpenes while squalene can give rise to triterpenes and steroids. Last, GGPP and GGPP can condense to form the 40-carbon phytoene structure which then can give rise to tetraterpenes.

Most plants contain so-called *essential oils*, which contain a mixture of volatile monterpenes and sesquiterpenes. Limonene is found in the essential oils of citrus peels while menthol is the chief monoterpene in peppermint essential oil (Figure 2.5). Two potentially nutraceutical diterpenes in coffee beans are kahweol and cafestol.[5,6] Both of these diterpenes contain a furan ring. As discussed by Miller and colleagues,[7] the furan ring component might be very important in yielding some of the potential antineoplastic activity of these compounds.

Several triterpenes (examples in Figure 2.6) have been reported to have nutraceutical properties. These compounds include plant sterols; however, some of these structures may have been modified to contain fewer than 30 carbons. One of the most recognizable triterpene families is the *limonoids*. These triterpenes are found in citrus fruit and impart most of their bitter flavor. Limonin and nomilin

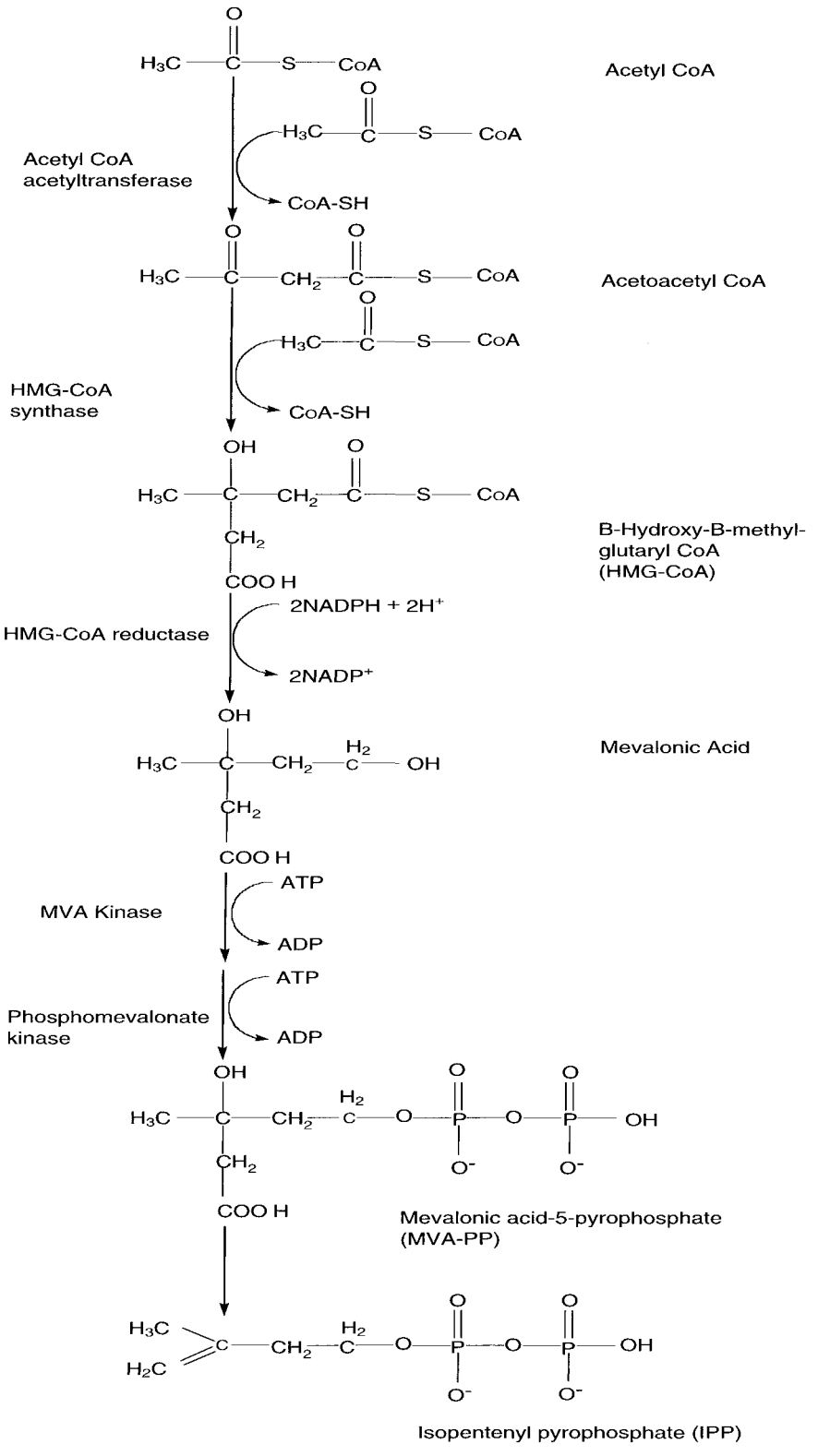


FIGURE 2.3 The mevalonic acid pathway.

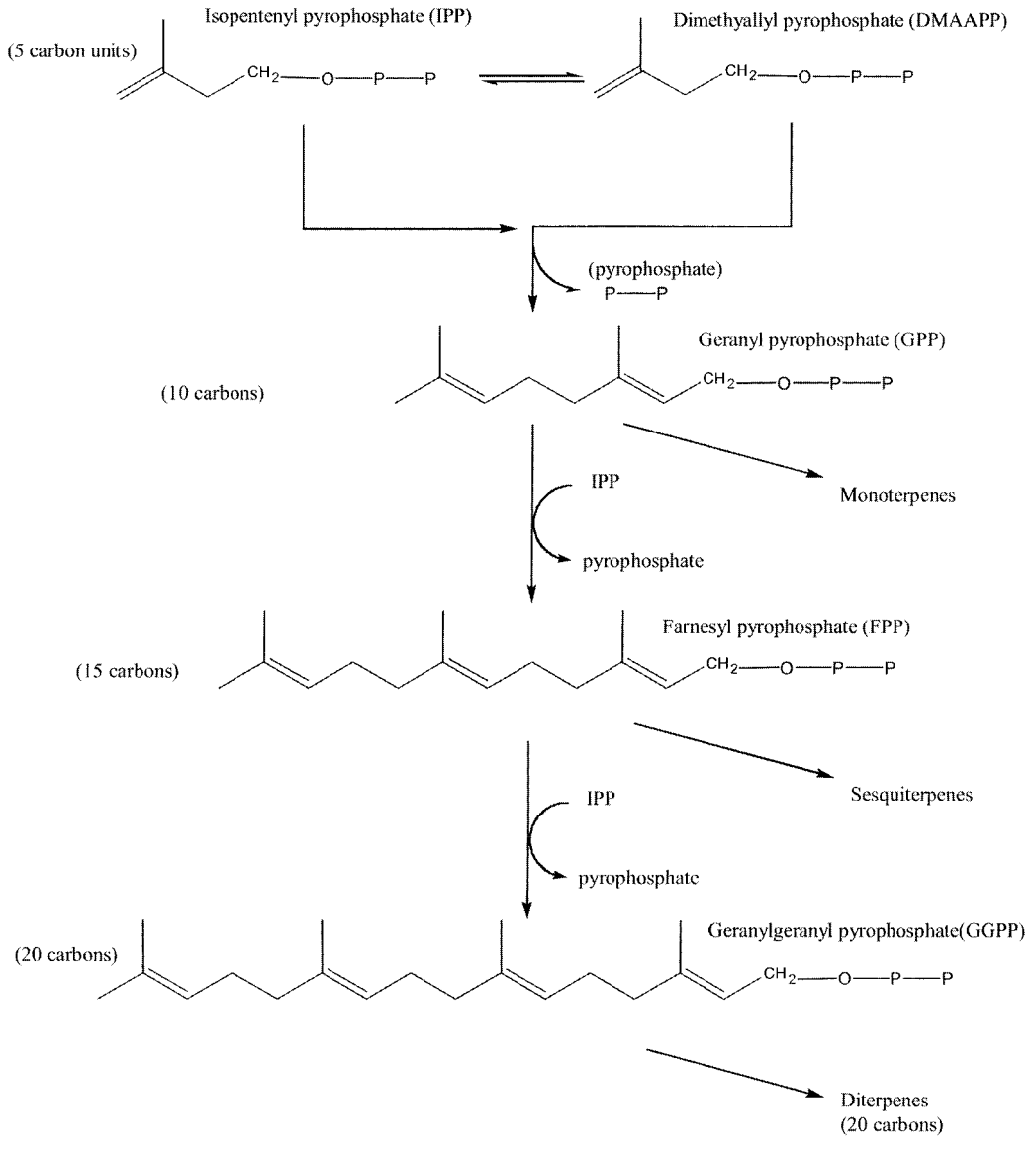


FIGURE 2.4 Formation of terpene structures. In addition: (1) FPP + FPP produces squalene (30 carbons) which yields triterpenes and steroids. (2) GGPP + GGPP produces phytoene (40 carbons) which yields tetraterpenes.

are two triterpnoids that may have nutraceutical application, limonin more so than nomilin.[7] Both of these molecules contain a furan component. In citrus fruit limonoids can also be found with an attached glucose, forming a limonoid glycoside.[8] As discussed above, the addition of the sugar group reduces the bitter taste tremendously and makes the molecule more water soluble. These properties may make it more attractive as a functional food ingredient. Saponins are also triterpene derivatives and their nutraceutical potential is attracting interest.[9–12]

The carotenoids (carotenes and xanthrophils), whose name is derived from carrots (*Daucus carota*), are perhaps the most recognizable form of coloring pigment within the isoprenoid class. As discussed by Southon and Faulks in Chapter 9, carotenes and xanthrophils differ only slightly

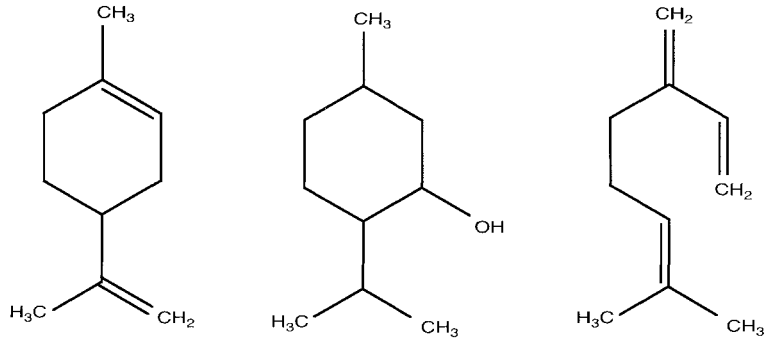
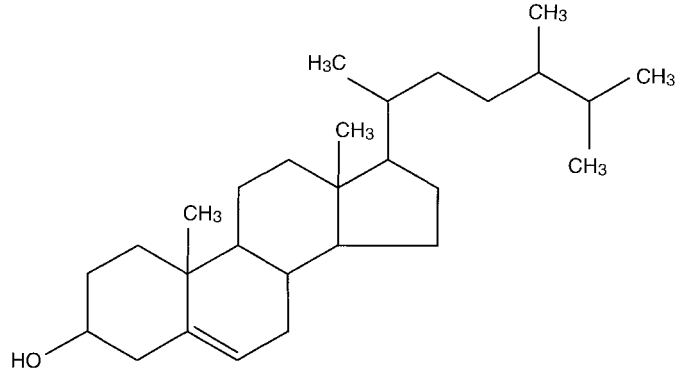


FIGURE 2.5 Structure of select monoterpenes.



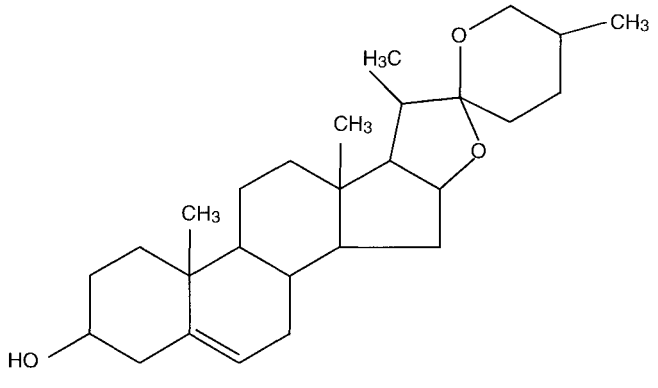


FIGURE 2.6 Examples of triterpenes.

in that true carotenes are purely hydrocarbon molecules (i.e., lycopene, α-carotene, β-carotene, γ-carotene); the xanthrophils (i.e., lutein, capsanthin, cryptoxanthin, zeaxanthin, astaxanthin) contain oxygen in the form of hydroxyl, methoxyl, carboxyl, keto, and epoxy groups. With the exception of crocetin and bixin, naturally occurring carotenoids are tetraterpenoids, and thus have a basic

structure of 40 carbons with unique modifications. The carotenoids are pigments that generally produce colors of yellow, orange, and red. Carotenoids are also very important in photosynthesis and photoprotection.

Different foods have different kinds and relative amounts of carotenoids. Also the carotenoid content can vary seasonal and during the ripening process. For example, peaches contain violaxanthin, cryptoxanthin, β-carotene, persicaxanthin, neoxanthin, and as many as 25 other carotenoids[13]; apricots contain mostly β-carotene, γ-carotene, and lycopene; and carrots contain about 50 to 55 parts per million of carotene in total, mostly α-carotene, β-carotene, ζ-carotene, as well as lycopene. Many vegetable oils also contain carotenoids with palm oil containing the most. For example, crude palm oil contains up to 0.2% carotenoids.

There are a few synthetic carotenoids, including β-apo-8′-carotenal (apocarotenal), and canthaxanthin. β-Apo-8′-carotenal (apocarotenal) imparts a light reddish orange color; and canthaxanthin imparts an orange red to red color.

B. Phenolic Compounds

Like the terpenoids, phenolic compounds are also considered secondary metabolites. The base for this very diverse family of molecules is a phenol structure, which is a hydroxyl group on an aromatic ring. From this structure, larger and interesting molecules are formed such as anthocyanins, coumarins, phenylpropamides flavonoids, tannins, and lignin. Phenolic compounds perform a variety of functions for plants including defending against herbivores and pathogens, absorbing light, attracting pollinators, reducing the growth of competitive plants, and promoting symbiotic relationship with nitrogen-fixing bacteria.

There are a couple of biosynthetic pathways that form phenolic compounds. The predominant pathways are the shikimic acid pathway and the malonic acid pathway. The shikimic pathway is more significant in higher plants; although the malonic acid is also present.[4] Actually, the malonic pathway is the predominant source of secondary metabolites in lower plants and fungi and bacteria. The shikimic pathway is so named because an intermediate of the pathway is shikimic acid. Inhibition of this pathway is the purpose of a commercially available herbicide (Roundup™).

The malonic acid pathway begins with acetyl CoA. Meanwhile in the shikimic pathway, simple carbohydrate intermediates of glycolysis and the pentose phosphate pathway (PPP) are used to form the aromatic amino acids phenylalanine and tyrosine. A third aromatic amino acid, tryptophan, is also a derivative of this pathway. As animals do not perform the shikimic acid pathway, these aromatic amino acids are diet essentials. Obviously, these amino acids are considered primary metabolites or products. Thus, it is the reactions beyond the formation of these amino acids that are really more related to the production of secondary metabolites. Once formed, phenylalanine can be used to generate flavonoids (Figure 2.7). The reaction that generates cinnamic acid from phenylalanine is catalyzed by one of the most-studied enzymes associated with secondary metabolites, phenylalanine ammonia lyase (PAL). The expression of PAL is increased during fungal infestation and other stimuli which may be critical to the plant.

From *trans*-cinnamic acid several simple phenolic compounds can be made. These include the benzoic acid derivatives vanillin and salicylic acid (Figure 2.8). Also, *trans*-cinnamic acid can be converted to *para*-coumaric acid. Simple phenolic derivatives of *para*-coumaric acid include caffeic acid and ferulic acid. CoA can be attached to *para*-coumaric acid to form *para*-coumaryl CoA. Both *para*-coumaric acid and *para*-coumaryl CoA can also be used to form lignin building blocks, *para*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol (see Chapter 17). After cellulose, lignin is the most abundant organic molecule in plants. To continue the formation of other phenolic classes, *para*-coumaryl CoA can undergo further enzymatic modification involving three malonyl CoA molecules to create polyphenolic molecules such as chalcones and then flavonones. The basic flavonone structure is then the precursor for the flavones, isoflavones, and flavonols. Also flavonones can be used to make anthocyanins and tannins via dihydroflavonols (Figures 2.7, 2.9, and 2.10).

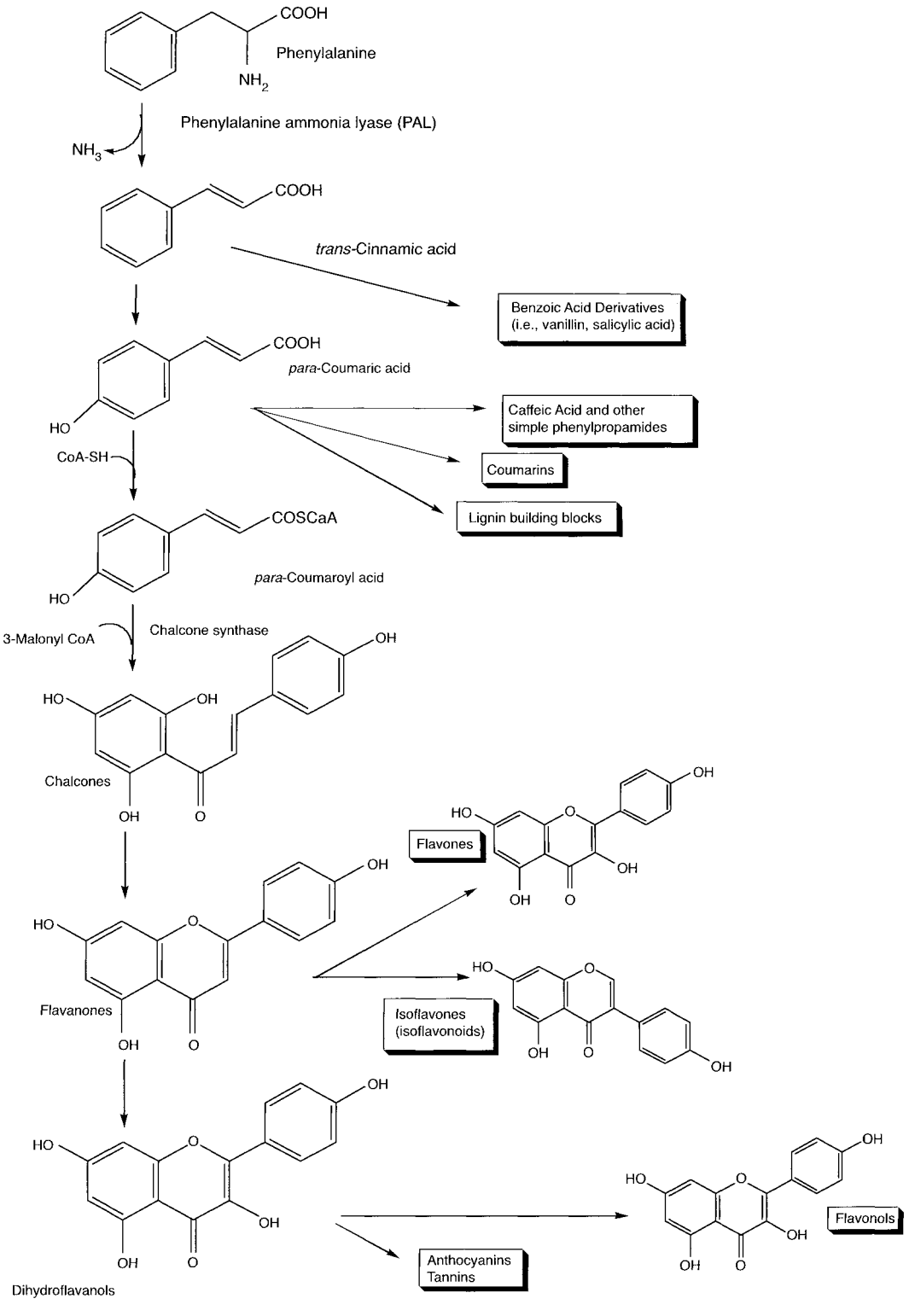
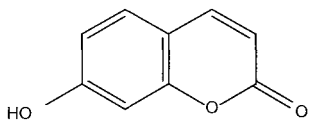
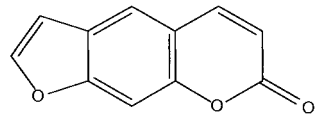


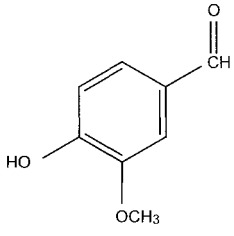
FIGURE 2.7 Production of plant phenolic molecules via phenylalanine. (Adapted from Waltenberg.[12])



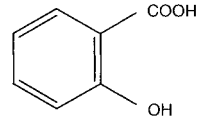
Umbelliferone
(a simple coumarin)



Psoralen
(a furanocoumarin)

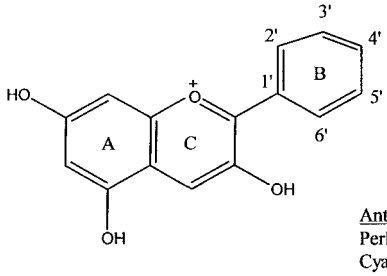


Vanillin



Salicylic acid

FIGURE 2.8 Select coumarins (first row) and two benzoic acid–derived phenolic molecules (second row).

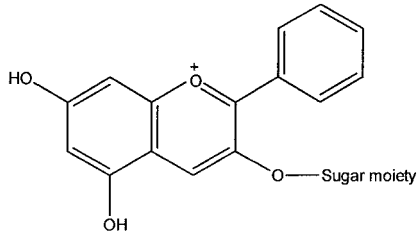


Anthocyanidin

Anthocyanidin Structures and Pigment Properties

Anthocyanidin	Substitutes	Color
Perlargonidin	4'-OH	Orange Red
Cyanidin	3'-OH, 4'-OH	Purplish red
Delphinidin	3'-OH, 4'-OH, 5'OH	Blue-Purple
Peonidin	3'-OCH3, 4-OH	Rose Red
Petunidin	3'-OCH_3, 4'-OH, 5'-OCH_3	Purple

A.



Anthocyanin

B.

FIGURE 2.9 Anthocyanidin (A) and molecular derivatives including anthocyanin (B).

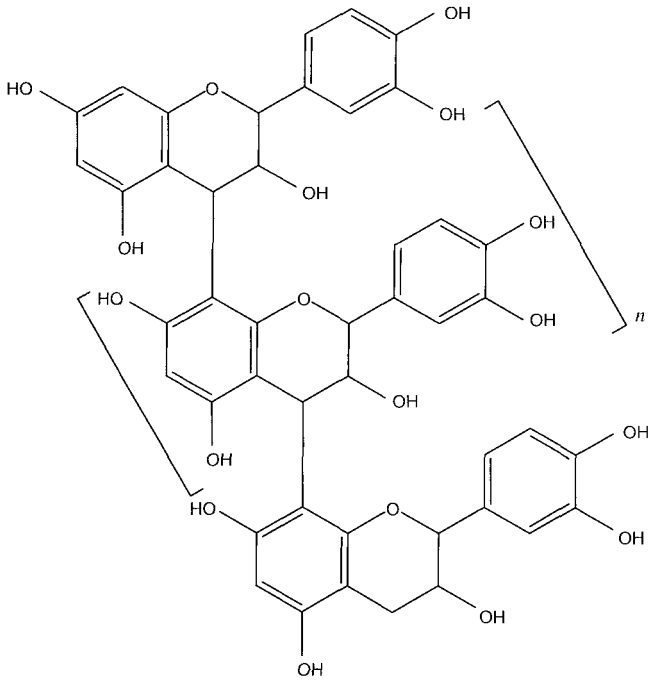


FIGURE 2.10 Basic tannin structure formed from phenolic units.

The flavonoids are one the largest classes of phenolic compounds in plants. The basic carbon structure of flavonoids contains 15 carbons and is endowed with 2 aromatic rings linked by a 3-carbon bridge (Figure 2.11).[4] The rings are labeled A and B. While the simpler phenolic compounds and lignin building blocks result from the shikimic pathway and are phenylalanine derivatives, formation of the flavonoids requires some assistance from both the shikimic pathway and the malonic acid pathway. Ring A is derived from acetic acid (acetyl CoA) and the malonic acid pathway (see the use of 3 malonyl CoA to form chalcones in Figure 2.7). Meanwhile, ring B and the 3-carbon bridge are derived from the shikimic acid pathway.[4] The flavonoids are subclassified

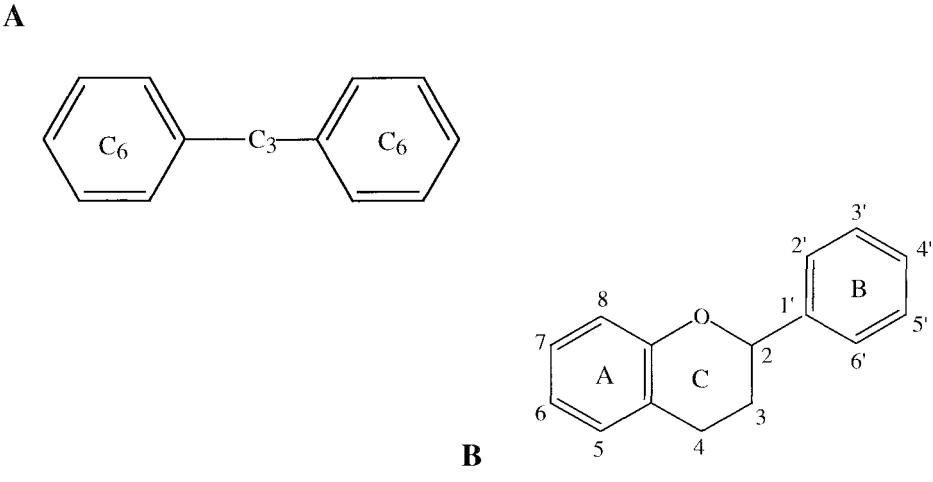


FIGURE 2.11 (A) Basic flavonoid carbon structure. (B) Flavonoid structure production: Carbons 5 to 8 are derived from the malonate pathway and 2 to 4 and 1′ to 6′ are derived from the shikimic acid pathway via the amino acid phenylalanine. Carbons 2 to 4 comprise the 3-carbon bridge.

based primairly on the degree of oxidation of the 3-carbon bridge. Also, hydroxyl groups are typically found at carbon positions 4, 5, and 7 as well as other locations. The majority of naturally occurring flavonoids are actually glycosides, meaning a sugar moiety is attached. The attachment of hydroxyl groups and sugars will increase the hydrophilic properties of the flavonoid molecule, while attachment of methyl esters or modified isopentyl units will increase the lipophilic character.

Anthocyanins and anthocyanidins (Figure 2.9) are produced by plants and function largely as coloring pigments. Basically, anthocyanins are anthocyanidins but with sugar moieties attached at position 3 of the 3-carbon bridge between ring A and B.[4] These molecules help attract animals for pollination and seed dispersal. They are responsible for the red, pink, blue, and violet coloring of many fruits and vegetables, including blueberries, apples, red cabbage, cherries, grapes, oranges, peaches, plums, radishes, raspberries, and strawberries. Only about 16 anthrocyanidins have been identified in plants and include pelargonidin, cyanidin, delphinidin, peonidin, malvidin, and petunidin.

Although the flavonols and flavones are structurally similar to their close cousin anthocyanidins and anthocyanidin glycoside derivatives anthocynanins, they absorb light at shorter wavelengths and thus are not perceived as color to the human eye. However, they may detected by insects and help direct them to areas of pollination. Because flavones and flavonols do absorb UV-B light energy (280 to 320 nm), they are believed to serve a protective role in plants. Also as discussed in more detail in the first chapter, certain flavonoids promote the formation of a symbiotic relationship between plant roots and nitrogen-fixing bacteria. The primary structural feature that separates the isoflavones from the other flavonoids is a shift in the position of the B ring. Perhaps the most ubiquitous flavonoid is quercetin. Hesperidin is also a common flavonoid especially in citrus fruit.

C. Carbohydrates and Derivatives

The glucose derivative ascorbic acid (vitamin C) is perhaps one of the most recognizable nutraceutical substances and is a very popular supplement. Ascorbic acid functions as a nutraceutical compound primarily as an antioxidant. Meanwhile, plants produce some oligosaccharides that may function as prebiotic substances, as discussed in Chapter 25.

Several plant polysaccharide families are not readily available energy sources for humans as they are resistant to secreted digestive enzymes. These polysaccharides are grouped together along with the phenolic polymer compound lignin to form one of the most recognizable nutraceutical families — fibers. By and large the role of fibers are structural for plants. For example, *cellulose* and *hemicellulose* are major structural polysaccharide found within plant cell walls. Beyond providing structural characteristics to plant tissue, another interesting role of certain fibers is in tissue repair after trauma, somewhat analogous to scar tissue in animals.

The nonstarch polysaccharides can be divided into homogeneous and heterogeneous polysaccharides as into well as soluble and insoluble substances (see Chapter 17). Cellulose is a homegeneous nonstarch polysaccharides as it consists of repeating units of glucose monomers. The links between the glucose monomers is β1-4 in nature. These polysaccharides are found in plant cell walls as microfibril bundles. Hemicellulose is found in association with cellulose within plant cell walls and is composed of a mixture of both straight-chain and highly branched polysaccharides containing pentoses, hexoses, and uronic acids. Pentoses such as xylans, mannans, galactans and arabicans are found in relatively higher abundance. Hemicelluloses are somewhat different from cellulose in that they are not limited to glucose and they are also vulnerable to hydrolysis by bacterial degradation. Another homopolysaccharide is pectin where the repeating subunits are largely methylgalacturonic acid units. It is a jellylike material that acts as a cellular cement in plants. The linkage between the subunits is also β1-4 bonds. The carboxyl groups become methylated in a seemingly random manner as fruit ripen. Chemically related to *pectin* is *chitin*. Chitin is not a plant polysaccharide but is found within the animal kingdom, but not necessarily humans.

It is a β1-4 homopolymer of *N*-acetyl-glucosamine found in shells or exoskeletons of insects and crustacea.[14] Chitin has recently surfaced as a dietary supplement.

Another family of polysaccharides that is worthy of discussion is *glycosaminoglycans* (GAGs). While these compounds are found in animal connective tissue, they are important to this discussion as they are potential components of functional foods. At present, GAG and chondroitin sulfate are popular nutrition supplements being used by individuals recovering from joint injuries and suffering joint inflammatory disorders. Glycosaminoglycans are often referred to as mucopolysaccharides. They are characterized by their content of amino sugars and uronic acids, which occur in combination with proteins in secretions and structures. These polysaccharides are responsible for the viscosity of body mucus secretions and are components of extracellular amorphous ground substances surrounding collagen and elastin fibers and cells of connective tissues and bone. Some examples of glycosaminoglycans are *hyaluronic acid* and *chondroitin sulfate*. Hyaluronic acid is a component of the ground substance found in most connective tissue including synovial fluid of joints. It is jellylike substance composed of repeating disaccharides of β-glucuronic acid and *N*-acetyl-*D*-glucosamine, hyaluronic acid can contain several thousand disaccharide residues and is unique from the other glycoaminoglycans in that it will not interact with proteins to form *proteoglycans*. Chondroitin sulfate is composed of β-glucuronic acid and N-acetylgalactosamine sulfate. This molecule has a relatively high viscosity and ability to bind water. It is the major organic component of the ground substance of cartilage and bone. Both of these polysaccharides have β1-3 linkage between uronic acid and acetylated amino sugars, but are linked by β1-4 covalent bonds to other polysaccharide units. Unlike hyaluronic acid, chondroitin sulfate will bind to proteins to form proteoglycans.

D. Fatty Acids and Structural Lipids

At this time, there are a couple fatty acids and/or derivatives that have piqued researchers interest for their nutraceutical potential. These include the ω-3 PUFA found in higher concentrations in plants, fish, and other marine animals and CLA produced by bacteria in the rumen of grazing animals such as cattle. The formation of CLA probably serves to help control the vitality of the releasing bacterial population in the rumen while plants and fish use ω-3 fatty acids for their properties in membranes. Some plants also use ω-3 PUFA in a second-messenger system to form jasmonic acid when plant tissue is under attack (i.e., by insect feeding).

The CLA precursor, linoleic acid, and ω-3 PUFA are produced largely in plants. In processes very similar to humans, plants construct fatty acids using two-carbon units derived from acetyl CoA. In humans and other animals, the reactions involved in fatty acid synthesis occur in the cytosol, while in plants they occur in the plastids. In both situations, FAS, acetyl CoA carboxylase enzymes, and acyl carrier protein (ACP) are major players. Plants primarily produce fatty acids to become components of triglycerides in energy stores (oils) as well as components of cell membrane glycerophospholipids and glyceroglycolipids which serve roles similar to the phopholipids in humans. In fact, several of the plant glycerophospholipids are generally the same as phospholipids. Some of the major fatty acids produced include palmitic acid (16:0), oleic acid (18:1 ω-9), linoleic acid (18:2 ω-6), and linolenic acid (18:3 ω-3). Grazing animals ingest linoleic acid which is then metabolized to CLA by rumen bacteria. Herbivorous fish also ingest these fatty acids when they consume algae and other seaweeds and phytoplanton. Carnivorous fish and marine animals then acquire these PUFA, and derivatives, from the tissue of other fish and marine life. Fish will further metabolize the PUFA to produce longer and more unsaturated fatty acids such as DHA (docosahexaenoic acid, 22:6 ω-3) and EPA (eicosapentaenoic acid, 20:5 w-3). The elongation and further unsaturation yields cell membrane fatty acids more appropriately suited for colder temperatures and higher hydrostatic pressures, usually associated with deeper water environments.

CLA is distinct from typical linoleic acid. First, CLA is not necessarily a single structure. There seem to be as many as nine different isomers of CLA. However, the primary forms are

mainly 9-*cis*, 11-*trans* and 10-*trans*, 12-*cis*. From these positions it is clear that the locations of the double bonds are unique. The double bonds are conjugated and not interrupted by methylene. Said another way, the double bonds are not separated by a saturated carbon but are adjacent. CLA is found mostly in the fat and milk of ruminant animals, which indicates that beef, dairy foods, and lamb are major dietary sources.

E. Amino Acid-Based

This group has the potential to include intact protein (i.e., soy protein), polypeptides, amino acids, and nitrogenous and sulfur amino acid derivatives. At this time a few amino acids are also being investigated for their nutraceutical potential. Among these amino acids is arginine, ornithine, taurine, and aspartic acid. Arginine has been speculated to be cardioprotective in that it is a precursor molecule for the vasodilating substance nitric oxide (NO).[15] Also arginine may reduce atherogenesis. Meanwhile the nonprotein amino acid taurine may also have blood pressure–lowering properies as well as antioxidant roles. However, the research in these areas is still inconclusive and the effects of supplementation of these amino acids on other aspects of human physiology is unclear. Several plant molecules are formed via amino acids. A few of the most striking examples, which are discussed in Chapters 11 through 13, are isothiocyanates, indole-3-carbinol, allyl sulfur compounds, and capsaicinoids. Another nutraceutical amino acid–derived molecule is folic acid, which is believed to be cardioprotective in its role of minimizing homocysteine levels.[16] Other members of this group would include the tripeptide glutathine and choline.

F. Microbes (Probiotics)

While the other groupings of nutraceuticals involve molecules or elements, probiotics involves intact microorganisms. This group largely includes bacteria and the criteria, as discussed by Farnworth in Chapter 25, are that a microbe must be resistant to acid conditions of stomach, bile and digestive enzymes normally found in the human gastrointestinal tract; able to colonize human intestine; safe for human consumption; and last have scientifically proven efficacy. Among the bacterial species recognized as having functional food potential are *Lactobacillus acidophilus*, *L. plantarum*, *L. casei, Bifidobacterium bifidum*, *B. infantis,* and *Streptococcus salvarius* subspecies *thermophilus*. Some yeasts have been noted as well, including *Saccharomyces boulardii.*

G. Minerals

Several minerals have been recognized for their nutraceutical potential and thus become candidates for functional food recipes. Among the most obvious is calcium with relation to bone health, colon cancer, and perhaps hypertension and cardiovascular disease. Potassium has also been purported to reduce hypertension and thus improve cardiovascular health. A couple of trace mineral have also been purported to have nutraceutical potential. These include copper, selenium, manganese, and zinc. Their nutraceutical potential is usually discussed in relation to antioxidation. Copper, zinc, and manganese are components of superoxide dismutase (SOD) enzymes while selenium is a component of glutathione peroxidase. Certainly more investigation is required in the area of trace elements in light of their metabolic relationships to other nutrients and the potential for toxicity.

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3 Isoprenoids, Health and Disease

Pamela L. Crowell and Charles E. Elson

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I. INTRODUCTION

Fruit, vegetables, and grains provide the foundation for dietary guidelines promulgated to reduce risk of cancer.[1-3] Fruit, vegetables and grains are uniquely rich sources of nondigestible carbohydrates and a group of micronutrients consisting of β-carotene, ascorbic acid, α-tocopherol, and folic acid. Contrary to the anticipated effects, clinical trials[4-10] and meta-analyses of the epidemiologic data[11] provide little evidence that these constituents uniquely provide chemoprevention. Fruit, vegetables, and grains also provide an array of non-nutritive phytochemicals with demonstrated potential as anticarcinogenic agents. Inhibitors of chemical carcinogenesis may be broadly classified either as blocking and suppressing agents.[12-15] Blocking agents act at the initiation phase of chemical carcinogenesis by preventing the formation of carcinogens from precursor substances and by preventing carcinogenic agents from reaching or reacting with critical target sites. Blocking agents include fiber, the antioxidant nutrients, the dithiolthiones, glucosinolates and indoles, isothiocyanates, flavonoids, phenols, and terpenes. Blocking actions include the induction of Phase I and II detoxifying enzymes, the dilution or binding of carcinogens in the gastrointestinal tract, and the

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capture of free radicals. Suppressing agents repress the expression of neoplasia in initiated cells. Suppressing agents include the protease inhibitors, phytosterols, allium compounds, and terpenes. These agents might provide substrates for the formation of antineoplastic agents or alter hormone status. Blocking and suppressing agents may have complementary, and occasionally overlapping, mechanisms of action.[15]

The initial findings that led to this discussion, namely, that the terpene, *d*-limonene, not only has both blocking[16,17] and suppressing[17] activities, but also causes the regression of frank tumors,[18] and work stemming from these findings has been reviewed.[19–31] Differing from most phytochemicals, the terpenes have efficacy in the suppression of the growth of a broad range of spontaneous tumors.

Terpenes are naturally occurring secondary products of plant mevalonate metabolism. Although terpenes have varying degrees of structural complexity, they consist only of multiples of the isoprene unit (Figure 3.1), e.g., monoterpenes (2x), sesquiterpenes (3x), diterpenes (4x), triterpenes (6x), tetraterpenes (8x), polyterpenes (nx), and steroids. The estimated 23,000 mevalonate-derived secondary products, differing in size, complexity, and function, but comprising repeating isoprene units, are known as isoprenoids. Related phytochemicals may be described as "mixed" isoprenoids: the prenylated coumarins, flavones, flavanols, isoflavones, chalcones, quinones, and chromanols, each with only a part of the molecule being derived via the mevalonate pathway.[32–36]

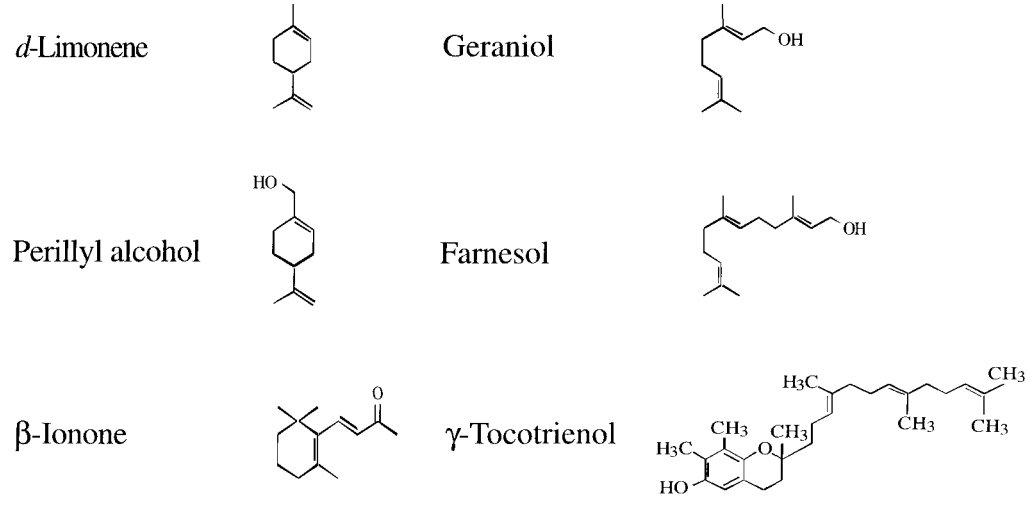

FIGURE 3.1 Structures of the isoprenoids discussed in this chapter; note relationships between *d*-limonene and perillyl alcohol, between geraniol and farnesol, and between farnesol and γ-tocotrienol.

Acyclic and cyclic isoprenoid alcohols consisting of two or three isoprene residues are emphasized in this chapter. These volatile isoprenoids have unique properties that contribute to the flavor and aroma profiles of herbs, spices, essential oils, and foods. Also discussed are isoprenoid-derived phytochemicals, β-ionone, and the tocotrienols, intakes of which parallel dietary intakes of β-carotene and vitamin E. Following are brief descriptions of the structural characteristics of these isoprenoids.[32–38] Data banks searched for sources of the specific isoprenoids reviewed in this chapter include Agricola, AGRIS, Biological Abstracts, Food Science and Technology Abstracts, and the Phytochemical & Ethnobiological Search Page provided by Leffingingwell and Associates.[39]

A. Geraniol and Farnesol

The ω-residue is the isoprene unit farthest from the hydroxyl groups of the acyclic isoprenoids, geraniol and farnesol (see Figure 3.1).[37] The designations cis and trans refer to the configuration of the residue across the double bond in the isoprene residue. That distinction applies to the

ω-residue only when one of the two methyl groups is substituted. The trivial names of the acyclic monoterpenoid alcohols with cis and trans bonds at the 2-carbon are, respectively, nerol and geraniol. There are four isomers of the sesquiterpenoid alcohol, namely, (1) 2-trans, 6-trans-, (2) 2-cis, 6-trans-, (3) 2-trans, 6-cis-, and (4) 2-cis, 6-cis-farnesol. The trivial names based on the ω-residue are, respectively, trans, trans-; trans, cis-; cis, trans-; and cis, cis-farnesol. Independent of differences in the bond configurations, the trivial names, myrcene and farnesene, geranial and farnesal, and geranoic acid and farnesoic acid, apply, in order, to hydrocarbon, aldehyde, and acid forms of the two isoprenoid alcohols.

Geraniol is a major component of the flavor profiles of basil, bay leaf, cardamom, coriander, dill, hop oil, lemongrass oil, mint, olive oil, rosemary, sage, and thyme. Food-related products containing geraniol include black currants, blueberries, carrots, grapes (and wine), oranges, strawberries, tea, and tomatoes.[39]

Farnesol is a major component of the flavor profiles of black pepper, cinnamon, clove, cumin, ginger, hops and hop oil, lavender, lemon verbena, lime oil, marjoram, olive oil, orange oil, oregano, and thyme. Food-related products containing farnesol include apples, beets, blueberries, citrus peel, grapes (and wine), mushrooms, spinach, strawberries, tea, and tomatoes.[39]

B. *d*-Limonene and Perillyl Alcohol

The structural diversity of the cyclic terpenes is more complex than that of the acyclic terpenes. Here the discussion is limited to two monocyclic monoterpenes, the hydrocarbon, *d*-limonene and the alcohol, perillyl alcohol. *d*-Limonene [(R)-(+)-limonene; *p*-mentha-1,8-diene] is the metabolic precursor of perillyl alcohol [(S)-(-)-perillyl alcohol].

Limonene is a major component of the flavor/aroma profiles of allspice, anise, bay leaf, bergamot, black pepper, caraway, cardamom, cinnamon, citrus peel oils, coriander, cumin, dill, fennel, ginger, hops and hop oil, lavender, lemon verbena, lime oil, mace, mint, nutmeg, orange oil, oregano, pecan oil, rosemary, sage, savory, spearmint, star anise, sweet basil, and thyme.[39]

Food-related products containing *d*-limonene include black currants, blueberries, carrots, celery, citrus fruit and beverages, colas, grapes (and wine), mushrooms, raspberries, and tea.[39]

Perillyl alcohol is a constituent of caraway, lavender oil, lilac oil, peppermint, sage, and spearmint.[39] *d*-Limonene is converted to perillyl alcohol by microbes[40,41] and rats.[42,43]

C. β-Ionone and β-Carotene

The primary volatile odor constituents derived from carotenoids are C13, C11, C10, and C9 derivatives formed via enzymatic oxidation or photo-oxidation of various carotenoids. β-Ionone, a C13 derivative, is the end-group analogue of β-carotene. Carotenoid-rich foods including apricots, beans, bell peppers, blackberries, blueberries, broccoli, carrots, cherries, citrus fruit, corn, grapes (and wine), mangos, parsley, peaches, plums, raspberries, strawberries, sweet potatoes, and tomatoes are sources of β-ionone.[39]

D. Tocotrienols and Tocopherol

The tocopherols have the general structure, 2-methyl-2-(4,8,12-trimethyltridecyl) chroman-6-ol. The naturally occurring stereoisomer, 2*R*, 4′*R*,8′*R* *d*-tocopherol ($R_1 = R_2 = R_3 = H$), is now termed *RRR*-tocopherol. The naturally occurring tocopherols also comprised *RRR*-α-tocophenol ($R_1 = R_2 = R_3 = CH_3$), *RRR*-β-tocophenol ($R_1 = R_3 = CH_3$, $R_2 = H$), *RRR*-γ-tocophenol ($R_1 = H$, $R_2 = R_3 = CH_3$), and *RRR*-δ-tocophenol ($R_1 = R_2 = H$, $R_3 = CH_3$). The tocotrienols have the general structure 2-methyl-2-(4,8,12-trimethyltrideca-3,7,11-trienyl) chroman-6-ol; continuing the discussion of the ω-residue (see farnesol), only *trans, trans* isomers of tocotrienol and α-, β-, γ-, and δ-tocotrienol occur in nature. Reduction of natural tocotrienols yields tocopherols with 2*R*,4′*R*,8′*R*, or 2*R*,4′*S*,8′*R*, or 2*R*,4′*S*,8′*S*, or 2*R*,4′*R*,8′*S* configuration; these stereoisomers are

termed 4′-*ambo*,8′-*ambo* tocopherols. Synthetic vitamin E, prepared without control of stereochemistry, consists of approximately equimolar proportions of four racemic pairs (eight diastereoisomers); formerly known as *dl*-α-tocopherol, it is now termed *all-rac*-α-tocopherol.[38]

Tocotrienols are constituents of high fiber cereals and grains (barley, oats, rice, and wheat) and oils extracted from olive and palm fruit.

II. ANTICANCER ACTIVITY

Geraniol, farnesol, *d*-limonene, perillyl alcohol, β-ionone, and γ-tocotrienol, the representative isoprenoids reviewed in this chapter, suppress tumor growth in animals exposed to direct-acting carcinogens as well as to precarcinogens, suppress the proliferation of cancer cells, and suppress the growth of implanted tumors.

A. Chemical Carcinogenesis

The isoprenoids emphasized in this review, geraniol,[44] *d*-limonene,[16–18,44–61] perillyl alcohol,[62–69] farnesol,[69] the tocotrienols,[70–75] and β-ionone[44,76] have been evaluated for chemoprotective activity. The isoprenoids, when administered by gavage or when incorporated into diets at levels ranging from 0.1 to 5%, suppress tumorigenesis initiated by direct-acting carcinogens as well as by precarcinogens that require activation. The carcinogens tested include 7,12-dimethylbenz[a]-anthracene (DMBA),[16–18,44–49] nitrosomethylurea (NMU),[49,50,68] *N*-ethyl-*N*-hydroxyethylnitrosamine (EHEN),[57] *N*-nitrosodiethylamine (NDEA),[55,61] benzopentaphene,[56] azoxymethane (AOM),[58,63,64] 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK),[51–54,60] *N*-nitrosobis(2-oxopropyl)amine (BOP),[59,62,68,69] 2-acetylaminofluorene (AAF),[74,75] diethylnitrosamine (DEN),[72,73] *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (MNNG),[60,61] and aflatoxin B1.[76] Target tissues for the carcinogens employed in these studies included the mammary gland,[16–18,44,46–50,70,71] lung,[51–54,56,63] liver,[57,72–76] colon,[58,66,67] stomach,[51,55,60,61] skin,[45] and pancreas.[59,66, 68,69] Some evaluations demonstrated efficacy when the isoprenoids were administered only during the initiation phase[17,46–48,54,67,72–74] and others when administered only during the promotion/progression phase[17,18,49,57,61–63,69] of chemical carcinogenesis.

Seminal findings realized from these studies follow. The isoprenoids, like other chemopreventive phytochemicals, induce blocking activities.[17,47,48,53,54] Beyond that response, isoprenoids act as suppressing agents[17,18,49,57,60–62,64,67,69] and, a seminal finding, cause the regression of chemically initiated tumors.[18,49,62,64,67,77] The aforementioned isoprenoids differ in chemopreventive efficacy. A comparison of the two cyclic monoterpenes shows that the efficacy of perillyl alcohol is double that of the hydrocarbon, *d*-limonene.[49,62] However, when administered to rats, dogs, and humans, *d*-limonene is converted to more active chemopreventive agents.[42,43,78–81] Geraniol, the acyclic monoterpene alcohol also has chemopreventive efficacy equal to that of *d*-limonene.[44] β-Ionone, the end-ring analogue of β-carotene, has greater efficacy than geraniol and *d*-limonene.[44] Within the group of isoprenoids discussed herein, γ-tocotrienol has the greatest chemopreventive efficacy.[72–75] The finding that α-tocopherol, a more potent antioxidant, attenuates the impact of γ-tocotrienol[82] reconciles the finding of a very modest chemopreventive impact when we fed a tocol blend containing about 30% α-tocopherol.[70,71]

B. Proliferation of Cancer Cells

Geraniol,[83–86] farnesol,[85–99] *d*-limonene,[86,100–107] perillyl alcohol,[86,102–116] β-ionone,[86,117–119] and the tocotrienols[86,119–125] suppress the proliferation of tumor cells. The growth-suppressive impact has been demonstrated with cancer cells grown in monolayer cultures. The growth of cancer cell lines as diverse as B16F10 melanoma cells,[83,86,116] MCF-7,[103,111,119,120,122,124] MDA-MB-231,[84,103] MDA-MB-435,[120,122,123,125] and T-47D[103] breast cells, HT-29,[102,106] SW480,[111] CT-26,[115] and Caco-2[119] colon

cells, HepG2 hepatoma cells,[101] WB-ras transformed epithelial cells,[105] KB oral epitheloid cells,[84] A54989,[98,106] and NCI-H226[118] lung cells, DU-14 prostate cells,[91] C-4-1,[91] Ishikawa,[118] HeLa11[121] and HeLaS3K[93] cervical cells, neuro 2A neuroblastoma cells,[114] and MIA PaCa-2,[85] B12/13,[109] and PANC-1104,[109] pancreatic cells is suppressed by isoprenoids. Also suppressed is the proliferation of cells grown in suspension culture, specifically, P388,[83,121] HL-60,[87,88,94,119] MD592,[90] NB4,[90] and CEM-C192,[99] leukemic cells, and PHA-stimulated leukocytes.[100] Physiologically normal cells respond to the growth-suppressive action of the isoprenoids but with much lower sensitivity. Normal cell lines tested include NIH 3T3,[107,110] CCD-18co colon,[119] CF-3,[91,93] foreskin fibroblasts, [93,118] hamster pancreatic ductal cells,[109] porcine aortic epithelial cells,[91] and prostate cells.[91]

As with the chemical carcinogen models, the selected isoprenoids differ in potency. This differential potency is reflected in the IC_{50} values (μmol/l), the concentration of an isoprenoid required to suppress the proliferation of B16F10 melanoma cells by 50%, calculated for *d*-limonene (450 ± 43), perillyl alcohol (250 ± 28), geraniol (150 ± 19), β-ionone (140 ± 23), farnesol (50 ± 4), and γ-tocotrienol (20 ± 3).[86] IC_{50} values reported the suppression of the growth of MIA PaCa2 pancreatic adenocarcinoma cells by perillyl alcohol (290 μmol/l), geraniol (265 μmol/l), and farnesol (39 μmol/l) reflect the same trend in efficacy.[85]

C. Suppression of the Growth of Implanted Tumors

The tumor implant model permits the *in vivo* evaluation of the impact an isoprenoid has on the growth of established tumors independent of a confounding effect on the initiation phase of chemical carcinogenesis. Two models apply to the transplant model. In one, diets containing isoprenoids are fed prior to and following tumor implant. Under this protocol, geraniol, 6.5, 23, and 130 mmol//kg diet, respectively, suppressed the growth of P388 leukemia cells[83] and B16F10 melanomas[126] implanted in C57BL mice, Morris 7777 hepatomas implanted in buffalo rats,[126] and PC-1 pancreatic adenocarcinoma cells implanted in Syrian Golden hamsters.[85] Farnesol (90 mmol/kg diet) and perillyl alcohol (263 mmol/kg diet) suppressed the growth of PC-1 pancreatic adenocarcinoma cells implanted in hamsters.[85] *d*-Limonene (370 mmol/kg diet) suppressed the metastatic growth of CT-26 tumor cells implanted in livers of mice.[115] In another test, the isomolar replacement of *dl*-α-tocopherol in the standard AIN-76A diet (116 μmol/kg diet) with γ-tocotrienol significantly suppressed the growth of B16F10 melanomas implanted in C57BL mice.[86] Farnesyl anthranilate (2 mmol/kg diet) also suppressed the growth of implanted B16F10 melanomas.[127]

The second implant model applies to chemotherapy as the isoprenoid treatment is introduced following the detection of an implanted tumor. Under these conditions, B16F10 melanomas responded to the growth-suppressive actions of β-ionone (2 mmol/kg diet)[3] and γ-tocotrienol (2 mmol/kg diet).[86] PC-1 pancreatic adenocarcinomas responded to the growth-suppressive actions of 89 mmol farnesol/kg diet, 130 mmol geraniol/kg diet, and 263 mmol perillyl alcohol/kg diet.[85, 112] The weight gain of host animals was not impaired by the isoprenoid inputs.[83,85,86,126]

III. PRELIMINARY CLINICAL APPLICATIONS OF ISOPRENOIDS

Phase I and Phase II clinical trials conducted in the United Kingdom tested oral administration of *d*-limonene at doses ranging from 0.5 to 12 g/m^2/day in patients with refractory solid tumors. *d*-Limonene chemotherapy resulted in one partial response in a breast cancer patient, and prolonged stable disease in three colorectal cancer patients.[128] Only mild gastrointestinal toxicity was observed. *d*-Limonene metabolites include perillic acid, dihydroperillic acid, *d*-limonene-1,2-diol, uroterpenol, and an isomer of perillic acid.[78–81] In addition, Nagourney[129] tested a combination of protein prenylation inhibitors (see below), namely, 2 g of orally administered *d*-limonene twice daily with two daily oral doses of 40 mg lovastatin. Two of nine patients with refractory solid tumors treated with the combination therapy exhibited partial responses of 11 and 5 months, and one patient who also received cisplatin and gemcitabine exhibited a complete response that persisted for 12 months.

Perillyl alcohol has been evaluated in patients with refractory cancers in a Phase I dose escalation trial conducted at the Wisconsin Comprehensive Cancer Center.[130] Administration of perillyl alcohol TID at 1600 mg/m^2/dose resulted in maximal plasma concentrations of the perillyl alcohol metabolites perillic acid and dihydroperillic acid,[62,130,131] and the drug half-life was approximately 2 h. Three patients with hormone-refractory prostate cancer had disease stabilization for 5 to 6 months while receiving perillyl alcohol chemotherapy, but no objective tumor responses were observed. Perillyl alcohol was well tolerated, and, as with *d*-limonene, mild gastrointestinal toxicity was noted. One ovarian cancer patient heavily pretreated with cytotoxic agents had grade 3 hematopoietic toxicity with perillyl alcohol, but it resolved upon withdrawal of the drug.

IV. POTENTIAL ANTITUMOR ACTIONS

These studies build on demonstrations of the concentration-dependent impact of isoprenoids on the growth of cultured tumor cells. Tumor growth represents, for this discussion, only an increase in cell population. Growth, resorting to a traditional nutritional term, reflects a "positive" balance between two factors, cell division and cell death; that is, the rate of division exceeds the rate of death. More definitive studies employing cell cycle analysis demonstrate that isoprenoids impact on both sides of the "balance" equation. Geraniol,[83] β-ionone,[119] γ-tocotrienol,[119] and perillyl alcohol[103,116] slow the progress of diverse lines of tumor cells through the cell cycle with a resultant buildup of cells in the G1 phase; other studies provide evidence that perillyl alcohol promotes the reversion of tumor cells to a physiologically normal state.[114] On the other side of the equation, these isoprenoids and farnesol initiate apoptotic cell death.[66,83,89–91,94–98,109,116,119]

The isoprenoids, like many other phytochemicals, have a modest activity that blocks carcinogenic agents from reaching or reacting with critical target sites. Extensive evaluations employing postinitiation chemical carcinogen models, *in vitro* assays with assorted tumor cell lines, tumor implant models, and Phase I and Phase II clinical trials provide concrete evidence of a more far-reaching tumor-suppressive action(s).

Actions attributed to the representative isoprenoids considered in this chapter include an antioxidant activity, the inhibition of phosphatidylcholine synthesis, actin cytoskeletal disorganization, activation/translocation of PKC-dependent signaling molecules, inhibition of protein prenylation, changes in the expression and/or localization of genes and gene products that modulate cell growth and cell death, and mevalonate starvation.

A. Antioxidant Activity

A putative membrane-targeted antioxidant activity[120,124,125,132] could account for the numerous findings that the tocotrienols suppress the growth of diverse lines of tumor cells.[86,119,120,122–125,132] That interpretation stands at odds with reports from the same laboratories that α–tocopherol has no impact on the proliferation of tumor cells.[119–125,132] Antioxidant activity is broadly associated with a blocking action during the initiation phase of carcinogenesis. α-Tocopherol, the more potent of the vitamin E active tocols, attenuates the impact of the tocotrienols on chemical carcinogenesis.[82] Also countering the postulation that antioxidant activity plays a role are findings that tumor cells exposed to the tocotrienols are arrested in the G1 phase of the cell cycle[119]; those cells that move through G1 undergo apoptotic cell death,[112,119,123] a response triggered by reactive oxygen species, a target for the antioxidant action. Also running counter to the antioxidant action is the finding that the 28-day weight of B16F10 melanomas implanted in host mice that were fed a modified AIN-76A diet, a diet with *d*-γ-tocotrienol substituted isomolarly for *dl*-α-tocotrienol, was 35% ($P < 0.05$) lower than that of tumors implanted in mice that were fed the AIN-76A diet.[86] The growth of tumors implanted in hosts fed an eightfold *dl*-α-tocopherol-enriched diet did not differ from that of the controls.[86] In summary, there is little basis for attributing the tumor-suppressive action of the tocotrienols to an antioxidant activity. This conclusion that the antitumor

action of the tocotrienols is not due to an antioxidant action applies to other isoprenoids with putative antioxidant activity, specifically lycopene and β-carotene. Intake of these isoprenoids has been shown to be inversely associated with cancer incidence in some but not all studies.[4–8,133,134] Chemically initiated carcinogenesis is likely blocked by the efficient singlet oxygen-quenching action of these isoprenoids[135–140] rather than to the induction of Phase I and Phase II detoxifying activities attributed to other isoprenoids.[17,47,48,53–55] Neither the singlet oxygen-quenching action[137–140] nor the induction of Phase I and Phase II detoxifying activities[17,47,48,53–55] explains the tumor-targeted growth-suppressive actions of diverse isoprenoids.

B. Phosphatidylcholine Synthesis, Actin Cytoskeletal Organization, and Translocation of PKC-Dependent Signaling Molecules

Of the isoprenoids considered in this chapter, farnesol has been shown to inhibit phosphatidylcholine synthesis which impacts on actin cytoskeletal organization, membrane-associated PKC and ras, and DAG signaling pathways.

An early study leading to the recognition that farnesol inhibits tumor growth was designed to assess the impact of a synthetic 2-*O*-methyl-lysophosphatidylcholine on the attachment of B16F10 melanoma cells to Matrigel. At nonlethal concentrations, that agent and *trans, trans*-farnesol, but not lysophosphatidylcholine, inhibited attachment.[92] Farnesol suppressed the growth of CEM-C1 leukemia cells without causing lysis. That inhibitory action was attenuated in the presence of either phosphatidylcholine or diacylglycerol.[141] That finding led to the recognition that a heat-stable, dialyzable constituent in the cytosol harvested from CEM-C1 cells incubated with farnesol, but not farnesol itself, inhibited choline phosphotransferase activity when added to cell homogenates.[142] Supporting studies reveal that CDP-choline accumulates, concomitant with the initiation of apoptosis, in farnesol-treated HL-60 cells.[87] The farnesol-mediated inhibition of phosphatidylcholine synthesis is associated with a time- and concentration-dependent response resulting in cell shrinkage and nuclear fragmentation with DNA laddering. The farnesol effect was partially reversed with supplemental phosphatidylcholine.[97] Farnesol additionally inhibits phospholipase C activity; supplemental diacyldiglyceride (DAG) reduced the extent of the farnesol-mediated cell cycle arrest and initiation of apoptosis.[96] Incubation with farnesol, a membrane-active lipid,[143] causes the translocation of protein kinase C (PKC) from membranes to the cytosol of neoplastic Hela S3K cells.[93] A farnesyl analogue, *S-trans*-farnesylthiosalicyclic acid, dislodges mature H-ras from the plasma membrane.[91] Accordingly, the farnesol-mediated arrest of neoplastic cells[99] might trace to farnesol-mediated effects on the DAG, PKC, or ras signaling system. PKC is not translocated in farnesol-treated non-neoplastically derived cells.[93] The recent finding of a saturable, high affinity binding site for farnesol on acute leukemia CEM C-1 cells,[99] if extended to other tumor cell lines, may provide an rationale for explaining the differential sensitivity between normal and neoplastic cells to the growth-suppressive actions of farnesol.[91]

Miquel et al.[89] provided confirmation that farnesol suppresses the synthesis of phosphatidylcholine; their findings differed in that farnesol directly inhibited microsomal choline phosphotransferase activity by competitively inhibiting the binding of DAG. The deficit in phophatidylcholine synthesis caused actin fiber and cytoskeleton disorganization. The resulting farnesol-induced apoptosis was preceded by the arrest of A549 lung adenocarcinoma cells in the G1 phase of the cell cycle. They subsequently reversed their conclusion that the cytoskeleton disorganization is independent of the farnesol-mediated inhibition of reductase activity[98] with the interpretation that a prenylated protein controls cell proliferation through the regulation of phosphatidylcholine synthesis.[89] One or another of the farnesol-mediated, tumor-sensitive impacts on DAG, PKC, and Ras signaling pathways may have relevance in determining why dietary farnesol suppresses the growth of implanted PC-1 pancreatic adenocarcinoma tumors but not that of the host hamster.[85]

C. Altered Gene Expression

The causative mechanisms responsible for cell cycle arrest, apoptosis, and other antitumorigenic effects of isoprenoids have been investigated by analysis of differential gene expression in untreated vs. isoprenoid-treated tumors, and by biochemical analyses of control and isoprenoid-treated tumor cells. Ariazi and Gould[116,144] identified a number of rat mammary carcinoma genes that were either increased or decreased in expression in response to *d*-limonene[144] or perillyl alcohol[116] chemotherapy. Increases in transforming growth factor β (TGF-β) pathway genes and proapoptotic genes were detected in isoprenoid-treated tumors. At the same time, decreases in the expression of genes encoding cyclin and cyclin-dependent kinases, and an increase in a cyclin-cdk inhibitor were detected in isoprenoid-treated tumors. Interestingly, these gene expression changes occurred only in the isoprenoid treated, regressing tumors and not in normal tissues, consistent with the antitumor activity and low toxicity of isoprenoids.[116]

The TGF-β pathway is upregulated in isoprenoid-treated liver[64] and mammary[116,144,145] tumors. TGF-β is activated from its latent form to its active form by the mannose-6-phosphate/insulin-like growth factor II receptor (M6P/IGFII-R). The M6P/IGFII-R receptor has a dual function in that it targets the mitogen insulin-like growth factor I for degradation. TGF-β binds to its receptor, TGF-β-R-II, which in turn binds and activates TGF-β-R-I. TGF-β-R-I then phosphorylates Smad2 and Smad3, which then bind Smad4. The Smad complexes then translocate to the nucleus to activate transcription of genes that induce apoptosis and inhibit cell cycle progression. Isoprenoid treatment increases the expression of most of these TGF-β pathway signaling molecules,[64,116,144,145] providing a plausible mechanism for the antitumor activity of isoprenoids in liver, mammary, and perhaps other tumor types.

D. Apoptosis

Interestingly, perillyl alcohol induces apoptosis in mammary,[116] pancreas,[109] and other tissue and cell types,[66,119] but the mechanisms are somewhat different in different tissues. In perillyl alcohol–treated rat mammary tumors, the expression of genes encoding the proapoptotic proteins Bax and Bad increases, while that of the antiapoptotic BCL-2 is unchanged.[116] On the other hand, perillyl alcohol treatment of pancreatic cancer cells causes an increase in expression of the proapoptotic protein Bak without affecting Bax or other related proteins.[109] The induction of Bax and Bad in mammary tumors is likely to be caused by the increased TGF-β pathway signaling.[116] This pathway is unlikely to account for the increase in Bak in isoprenoid-treated pancreatic tumor cells, however, because the Smad4 (DPC4) gene is deleted or otherwise mutated in most pancreatic cancers,[146] and pancreatic cancer cells are insensitive to TGF-β.[147]

E. Protein Prenylation

Limonene and perillyl alcohol inhibit the post-translational prenylation of proteins with a farnesyl or geranylgeranyl moiety[148] (Figure 3.2) in many cell types.[79,100–102,107,108,110,149,150] These effects are likely to be due to the ability of isoprenoids to inhibit directly the prenyl-protein transferases that catalyze these reactions (Figure 3.2).[81,151] The prenylated proteins affected by the isoprenoids include Ras and Ras-related proteins.[79,100–102,107,108,110,149,150] In some cell types, isoprenoids cause a decrease in Ras expression rather than a decrease in its prenylation.[104,152] Ras is not affected by the isoprenoids in every cell type,[104,105,108] implicating proteins other than Ras[148,152–156] such as nuclear lamin B[119] or prenylation-independent mechanisms in the antitumor activity of isoprenoids.

F. Mevalonate Starvation

The early work leading to recognition of the tumor-suppressive properties of the isoprenoids has been summarized in recent reviews.[19–21,25–29] That work initially focused on certain aspects of

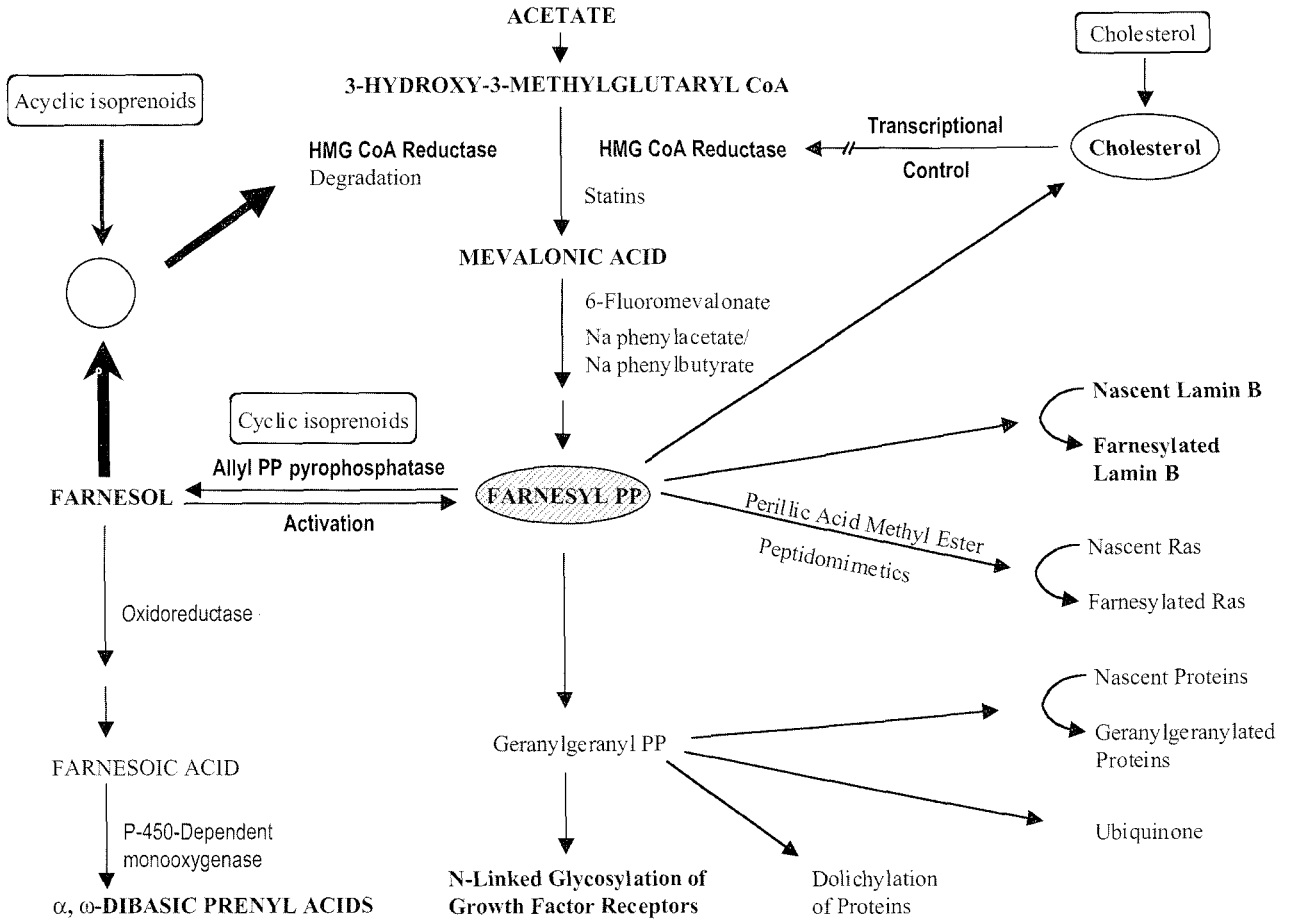

FIGURE 3.2 An orientation to the mevalonate pathway with delineation of prospective role of farnesol as a secondary regulator of mevalonate synthesis, farnesol oxidation, mevalonate-derived products essential for cell survival, sites of pharmacological intervention, and prospective sites of acyclic and cyclic isoprenoid-mediated actions.

cholesterol metabolism.[25–29] The salient points from a recent review[29] provide a basis for the following discussion (see Figure 3.2). In animal cells, a finely tuned system maintains both a pool of isoprenoid pathway intermediates essential for cell survival and cholesterol homeostasis. Cholesterol, the bulk end product in sterologenic tissues, exerts transcriptional control on sequential activities in the pathway (Figure 3.2). 3-Hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase, the rate-limiting activity in the isoprenoid pathway, catalyzes the reductive synthesis of mevalonate. Its regulation additionally integrates regulatory activities at translational and post-translational levels. The latter, a secondary level of control active when cellular isoprenoid requirements are satisfied, is mediated by a nonlysosomal cysteine protease (Figure 3.2). This protease activity, a process accelerated by farnesol, has high specificity for HMG CoA reductase.[157–164] Among the discrete microsomal allyl pyrophosphate pyrophosphatase activities, one with a high specificity for farnesyl pyrophosphate may play a critical role in diverting farnesol from the isoprenoid pathway (Figure 3.2).[157–166] Farnesol has a short biological half-life. On the one hand, it may be oxidized by cytosolic and microsomal activities and excreted as α, ω-dibasic prenyl acids[167–172] and, on the other, reactivated by sequential CTP and ATP kinase activities in the endoplasmic reticulum (Figure 3.2).[173,174]

Competitive inhibitors of HMG CoA reductase, collectively statins, have a marked impact on cell proliferation; this effect was initially attributed to a rate-limiting pool of cholesterol with a concomitant attenuation of membrane assembly (Figure 3.2). The seminal finding of the incorporation of mevalonate-derived products into animal proteins[175] suggested an alternative rationale for explaining the impact of statins on cell proliferation. Investigators first identified the carboxyterminal cysteine residue of lamin B[176–178] and immediately thereafter that of p21ras[179–181] as the site where a farnesyl group is covalently attached. The CaaX carboxy-terminal sequence motifs of these proteins direct, in sequence, the transfer of the farnesyl moiety from farnesyl pyrophosphate to the cysteine sulfhydryl, the proteolysis of the terminal aaX amino acids, and carboxymethylation of the COOH-terminal farnesylated cysteine.[176–187] This processing is essential for the biological function of the nuclear lamins[188–191] and virtually all members of the ras superfamily of proteins.[179–188] The statin-mediated suppression of mevalonate synthesis suppresses ras farnesylation as well as isoprenylation of other proteins localized in the plasma and subcellular membranes.[158] As a consequence of mevalonate starvation, cells incubated with statins tend to accumulate in the G1/S phase of the cell cycle[119,192–194] or undergo apoptotic death.[119,195–201] These effects are reversed with supplemental mevalonate but not with cholesterol. Dose-limiting toxicities, rhabdomyolysis, nausea, diarrhea, and fatigue preclude the widespread application of high-dose statins as chemotherapeutic agents.[202] Other agents targeting the mevalonate pathway (Figure 3.2), specifically sodium phenylacetate, sodium phenylbutyrate, and 6-fluoromevalonate, may have efficacy in the control of tumor growth.[203–211] The aforementioned agents deprive cells of one of the substrates for the isoprenylation reactions. The transfer of the farnesyl moiety of farnesyl pyrophosphate to the carboxy-terminal cysteine of oncogenic ras offers a second target for pharmacological intervention (Figure 3.2).[19–24,78,79,102,107,108,149,151,212–214]

The aforementioned approaches to cancer chemotherapy are not targeted to tumor cells. An early recognized, tumor-specific aberration in the regulation of HMG CoA reductase,[215] might offer an opportunity for tumor-targeted intervention.[25–29] Reductase activity in essentially all tumors and in carcinogen-treated livers is elevated and resistant to sterol-mediated feedback regulation (Figure 3.2).[216–226] The opportunity for intervention lies in findings that the sterol feedback-resistant tumor HMG CoA reductase retains high sensitivity to post-transcriptional regulation,[224] that is, the regulatory actions triggered by farnesol[163–165] and farnesyl homologues, e.g., the tocotrienols (see Figure 3.1),[227] farnesyl acetate,[228] and ethyl farnesyl ether.[228] The sensitivity of HMG CoA reductase activity in tumor tissues to farnesol-mediated downregulation is several-fold greater than that of reductase activity in sterologenic tissues.[29,227] This may explain the very marked impact acyclic isoprenoids have on tumor growth, the absence of an effect on the growth of animals and the very modest impact on serum cholesterol levels.[229–233] Here, it is noted that in some studies, the tocotrienols did

not lower serum cholesterol.[234–236] In two of those studies, HMG CoA reductase, the target of the tocotrienol action, was fully suppressed by dietary inputs of cholesterol (Figure 3.2).[234,235] In the third,[236] the tocol blend consisted of 33% α-tocopherol, a level amply sufficient to attenuate[82] the weak reductase-suppressive action of α-tocotrienol.[231]

The cyclic isoprenoids reviewed herein also suppress cholesterol synthesis.[237–241] An early report suggested the cyclic isoprenoids trigger the proteolytic degradation of HMG CoA reductase.[237,238] Whereas the farnesol-mediated impact on reductase level is detected within minutes, the cyclic isoprenoid effect develops over a 2 to 3 h interval. The triggering thus appears to be a secondary response to the cyclic isoprenoid-mediated activation of farnesyl pyrophosphate pyrophosphatase[165,242] with the proteolytic action being initiated by farnesol (see Figure 3.2).[163–165]

Limonene and perillyl alcohol inhibit the incorporation of the mevalonate-labeled farnesyl moiety into 21- 26-kDa proteins.[107] β-Ionone similarly inhibits the incorporation of the mevalonate-labeled farnesyl moiety into lamin B.[119] These results suggest that the cyclic isoprenoids inhibit farnesyl protein transferase; however, *d*-limonene and perillyl alcohol are weak inhibitors, *in vitro*, of the activity.[151] This discrepancy might be explained in that a common metabolite, perillic acid methyl ester, competes with farnesyl pyrophosphate for binding to the transferase.[151] An alternative rationale argues that the cyclic isoprenoid-mediated induction of farnesyl pyrophosphate pyrophosphatase depletes cells of the farnesylation substrate.

Recapping this discussion, acyclic isoprenoids trigger the degradation of HMG CoA reductase, the rate-limiting step in the synthesis of farnesyl pyrophosphate. Cyclic isoprenoids induce an allyl pyrophosphate pyrophosphatase activity that depletes cells of one the substrates, farnesyl pyrophosphate, which is required for the post-translational modification of ras, the nuclear lamins, and other proteins essential for cell proliferation; farnesol then secondarily triggers the degradation of HMG CoA reductase (Figure 3.2). Some of the findings support this concept. Supplemental mevalonate reverses the statin- and acyclic-mediated suppression of cell proliferation[83] but not that of the cyclic isoprenoid-mediated suppression (Mo and Elson, unpublished). When tested in blends, the growth suppressive actions of acyclic isoprenoids are additive, the actions of cyclic isoprenoids are additive; when presented in a common blend, the actions of acyclic and cyclic isoprenoids are, as predicted by their separate sites of action (see Figure 3.2), synergistic.[27,29,119]

γ-Tocotrienol, a *trans,trans*-farenesylated tocol analogue,[86,227,231] farnesylamine,[243] farnesyl acetate,[228,244] farnesylthiosalicylic acid,[91,244,245] menaquinone-3,[246] and ethyl farnesyl ether[228] suppress cell proliferation and/or HMG CoA reductase activity with substantially greater efficacy than farnesol. These farnesyl homologues, it is suggested, have a relatively extended cellular half-life compared with farnesol.[127] Geraniol and the geranylated tocol analogue suppress HMG CoA reductase activity also but with lower potency, respectively, than farnesol and γ-tocotrienol.[227,231] The acyclic monoterpene pair, geraniol (*trans*) and nerol (*cis*), differ substantially in the potency of their tumor-suppressive actions whereas those of *trans, trans*-farnesol and *trans,cis*-farnesol are equally potent (Reference 127 and unpublished observations). These findings point to the importance of the chain length and the configuration of the 2-position double bond in determining the potency of the acyclic isoprenoids. Cyclic isoprenoids also differ substantially in their tumor-suppressive[22,27,81,86,100,107,150,151] and reductase-suppressive[237] potencies. The hydrocarbon, *d*-limonene is less potent than both its oxygenated metabolites and the naturally occurring ketones and aldehydes derived from *d*-limonene.

V. SUMMARY

Geraniol, farnesol, *d*-limonene, perillyl alcohol, β-ionone, and γ-tocotrienol, the representative isoprenoids reviewed in this communication, suppress tumor growth in animals exposed to direct-acting carcinogens as well as to precarcinogens. These isoprenoids also suppress, with some degree of specificity, the proliferation of tumor cells. That suppression involves two actions, the arrest of the tumor cells in the G1 phase of the cell cycle and the initiation of apoptotic cell death. Further,

these isoprenoids markedly suppress the growth of implanted tumors but have little effect on host animals. A potential antioxidant activity attributed to these structurally diverse isoprenoids, some having kinship with more powerful dietary antioxidants, does not explain their tumor-suppressive action. Other candidate mechanisms through which these and a massive number of other isoprenoids might suppress the growth of tumors are reviewed.

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4 Isoflavones: Source and Metabolism

Suzanne Hendrich and Patricia A. Murphy

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I. INTRODUCTION TO SOY AND ISOFLAVONES

Soybeans are a major source of phytochemicals. In addition to its high-quality protein, other soybean components have been identified as having health protective effects including phytoestrogenic isoflavones,[1] saponins,[2] sphingolipids,[3] phenolic acids,[4] and Bowman–Birk trypsin inhibitor.[5] Isoflavones from soybeans and several other legumes are classified as phytoestrogens due to their weak estrogenic activity (100,000 < β-estradiol) in mammalian systems.[6] Genistein, daidzein, and glycitein are the aglycone isoflavonoids in soybeans. Formononetin and biochanin A are the principal aglycones in alfalfa, clover, and chickpeas. The phytoestrogenic coumestan, coumesterol, is found in germinated soybeans and in alfalfa and clover seeds. However, coumesterol is virtually absent from dry soybean seeds and foods made from these seeds. The isoflavones occur predominately as β-glucoside forms in plants and foods derived from these plants. Soybeans and soy foods can contain β-glucosides, 6″-*O*-malonylglucosides, and 6″-*O*-acetylglucosides in addition to the aglycones (Figure 4.1). Although many plants synthesize isoflavones, the estrogenic isoflavones are confined to a few plant foods consumed by humans. The development of a database on isoflavone levels in human foods has been simplified by segregation of isoflavones into a few food plant species.

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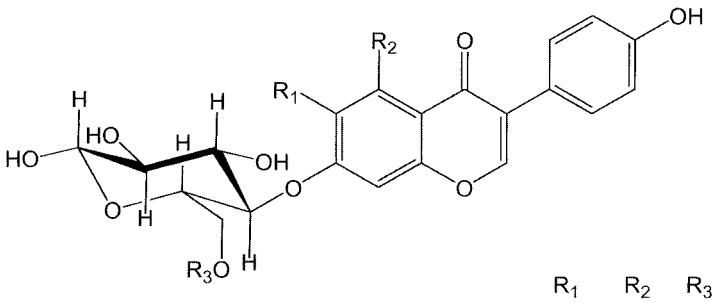

	R_1	R_2	R_3
Genistin	H	OH	H
Glycitin	OCH_3	H	H
Daidzin	H	H	H
6"-O-Malonyl			$OCCH_2COOH$
6"-O-Acetyl			OCH_3

FIGURE 4.1 Isoflavones.

This database is now available at *http://www.nal.usda.gov/fnic/foodcomp/Data/isoflav/isoflav.html* as downloadable files.

A. Analysis of Isoflavones

Analysis of isoflavones in foods and plants requires use of authentic standards for quantification and identification. The availability of these compounds has been a barrier for research in this field. Commercial sources of these components have been limited, until recently, to the aglycones. The β-glucosides became available recently. However, the malonylglucosides and acetylglucosides must be purified by investigators. The malonyl forms will self-decarboxylate within a matter of hours in solution after extraction from the soy matrix. Many researchers use molecular weight adjustment factors to quantify the isoflavone forms that they do not have available using relative retention times from high-performance liquid chromatography (HPLC) and different ultraviolet (UV) absorbance patterns for the different forms (Table 4.1) There are several simple routes of synthesis of the isoflavone aglycones.[7,8] More complex synthetic routes are also known.[9] Typically, the isoflavone standards are purified from the plant material or from the metabolite source such as urine by a variety of column chromatography steps involving Sephadex LH-20, silica, and C18 chromatography both in classic open columns or semipreparative HPLC with alcoholic or acetonitrile solvents. Recrystallization from ethanol after chromatography can improve purity. The major limitation to scaling up purification is the rather low solubility of the isoflavones even in alcoholic solvents.

After the isoflavones have been separated, their identity and purity should be authenticated by at least three techniques. Authentication can be accomplished by several physical chemistry techniques including UV spectra and bathochromic shifts,[10] melting points, HPLC retention time, mass spectral analysis, nuclear magnetic resonance (NMR) spectroscopy, and infrared (IR) spectroscopy.

Isoflavones have UV spectra that can be used for HPLC peak identification using diode array detectors. Additionally, the extinction coefficients at λ_{max} wavelengths can be used to calibrate standard solutions (see Table 4.1). However, there is no universal agreement in the literature for the extinction coefficients for most of the isoflavones. The extinction coefficients must be standardized if all laboratories are going to evaluate concentrations of individual isoflavone forms without isolating and purifying each one. The concentration ranges for standards used in Murphy's laboratory are listed in Table 4.1. Isoflavone melting points and molecular weights (Table 4.1) are also needed for the standardization of soy isoflavone measurements, commonly employing UV absorbance spectra and appropriate isoflavone standards.

TABLE 4.1
Melting Point, Molecular Weights, UV Absorbance Maxima, and Molar Extinction Coefficients of Soybean Isoflavones

Compound	Molecular Weight	Melting Point (°C)	λ_{max} (nm)	Extinction Coefficient (ε)	Working Range (μg/g)
Daidzein	254	323[a]	249[a]	31563[a]	5–200
		290[b]	249[c]	26915[c]	
		323[d]	250[e]	20893[e]	
			252[f,g]	18197[f,g]	
			250[h]	26000[h]	
			252[i]	25400[i]	
Daidzin	416	234[a]	249[a]	26830[a]	5–200
			254[h]	29000[h]	
Acetyldaidzin	458	196[f]	256[a]	29007[a]	
			252[f,g]	16331[f,g]	
Malonyldaidzin	502	168[j]	258[a]	26830[a]	
			258[j]	31622[j]	
Genistein	270	301[a]	263[a]	35323[a]	5–200
		291[f]	263[c]	31622[c]	
		298[d]	263[e]	37154[e]	
			261[f,g]	16331[f,g]	
			254[h]	37300[h]	
			252[j]	25400[j]	
Genistin	432	256[a]	263[a]	30895[a]	5–200
			254[h]	41700[h]	
Acetylgenistin	474	186[g]	261[c]	38946[a]	
			261[f,g]	32358[c]	
				18197[f,g]	
Malonylgenistin	518	145[q]	260[a]	30895[a]	5–100
			260[j]	26303[j]	
Glycitein	285	338[k,l]	256[m]	25388[a]	5–100
		311–313[o]		22387[n]	
Glycitin	447	192–195[m]	259[n]	26713[a]	5–100
Acetylglycitin	489	—	260[t]	29595[a]	
			259[q]	26303[p]	
				25120[q]	
Malonylglycitin	533	162[q]	260[p]	26303[a,p]	
			260[j]	32360[j]	

[a] Values Murphy has determined or used with isolated in-house standards.

[b] See Reference 7 in text.

[c] Weast, R.C., *Handbook of Physics and Chemistry*, 51st ed., The Chemical Rubber Co., Cleveland, OH, 1970.

[d] Gyorgy, P., Murata, K., and Ikehata, H., Antioxidants isolated from fermented soybeans (tempeh), *Nature*, 203, 870, 1964.

[e] Ollis, W.D., The isoflavonoids, in *The Chemistry of Flavanoid Compounds*, Geissman, T.A., Ed.; Macmillan, New York, 1962, 353.

[f] Ohta, N., Kuwata, G., Akahori, H., and Watanabe, T., Isoflavonoid constituents of soybeans and isolations of a new acetyl daidzin, *Agric. Biol. Chem.*, 43, 1415, 1979.

[g] Ohta, N., Kuwata, G., Akahori, H., and Watanabe, T., Isolations of a new isoflavone acetyl glucoside, 6″-O-acetyl genistin, *Agric. Biol. Chem.*, 44, 469, 1980.

[h] See Reference 11 in text.

[i] See Reference 18 in text.

continued

TABLE 4.1 (CONTINUED)
Melting Point, Molecular Weights, UV Absorbance Maxima, and Molar Extinction Coefficients of Soybean Isoflavones

[j] Kudou, S., Fleury, Y., Welti, D., Magnolato, D., Uchida, T., Kitamura, K., and Okubo, K., Malonyl isoflavone glycosides in soybean seeds (*Glycine max* Merrill), *Agric. Biol. Chem.*, 55, 2227, 1991.

[k] Reference 15 in text.

[l] Nogradi, M. and Szollosy, A., Synthesis of 4′,7-dihydroxy-6-methoxyisoflavone 7-O-β-*D*-glucopyranoside (Glycitin), *Liebigs. Ann.*, 1651, 1996.

[m] See Reference 46 in text.

[n] Naim, M., Gestetner, B., Kirson, I., Birk, Y., and Bondi, A., A new isoflavone from soya beans, *Phytochemistry*, 12, 169, 1973.

[o] Naim, M., Gestetner, B., Kirson, I., Birk, Y., and Bondi, A., Antioxidative and antihemolytic activity of soybean isoflavones, *J. Agric. Food Chem.*, 22, 806, 1976.

[p] Taken from Glycitein.

[q] Kudou, S., Shimoyamada, M., Imura, T., Uchida, T., and Okubo, K., A new isoflavone glycoside in soybean seeds (*Glycine max* Merril), Glycitein-7-O-β-D-(6′-O-acetyl)-glucopyranoside, *Agric. Food Chem.*, 55, 859, 1991.

Isoflavones (and other flavanoids) have characteristic bathochromic shifts from the oxidation of specific hydroxyl groups by specific reagents.[10] The 7-hydroxyl of isoflavones can be oxidized by sodium methoxide. The 4′- and 3′-hydroxyls are oxidized in the presence of sodium acetate. The 5-hydroxyl can be oxidized by aluminum trichloride in HCl. Mabry and colleagues[10] present a spectral library for many flavanoids and isoflavanoids.

Melting points (Table 4.1), although an old technique, provide unequivocal confirmation of the purity of an isoflavone, and occasionally may be useful for compound identification. A single HPLC peak may be detected and scanned as a purportedly pure compound that yields a broad and low temperature thermogram indicating impurities. Melting points can confirm the purity of commercial standards.

Mass spectral (MS) analysis, using one of several types of MS analysis, can yield information about mass and purity. NMR and IR spectroscopy can provide confirmatory information on the chemical structure of the isoflavone, although this instrumentation is not yet routinely available in most laboratories.[11,12]

Analysis of aglycone isoflavones can be accomplished by gas chromatography[13,14]; however, HPLC analysis is the method of choice to determine the native distribution of isoflavone glucosides found in food and plant material. Isoflavone detection can be accomplished with single-wavelength UV absorbance or diode array detectors,[15,16] electrochemical detectors,[17] or using LC-MS interfaces, a mass detector, or a mass spectrometer.[12,18,19] For most laboratories, one of the UV absorbance techniques is suitable for the concentrations of isoflavones found in foods. Capillary electrophoresis with UV diode array detection has been reported for isoflavone analysis.[20]

HPLC analysis of isoflavones can be designed to be simple to perform routinely, but requiring considerable effort to establish, or it can be designed to be more labor-intensive for each analysis but easy to set up. The authors have selected the former. The isoflavone forms have been isolated to establish standard curves, response factors, and retention times with their HPLC system.[21] Routine samples are extracted in 20 ml acetonitrile, water (dependent on sample matrix), and 5 ml of 0.10 *N* HCl for 2 h, filtered, concentrated, and loaded onto an autosampler for HPLC analysis. The concentrations of all forms are obtained from injection of a single sample.

The alternative HPLC approach has involved acid hydrolysis in 1 to 2 *M* HCl of the soybean or soyfood for 1 to 3 h, followed by extraction of aglycones, concentration, and HPLC analysis using only aglycone standards. The results are either "total" isoflavones [22] or, "free" and "conjugated" forms if aglycones are first assayed; then the sample is hydrolyzed and reassayed.[23]

Extraction conditions must be optimized for the sample matrix being assayed. Extraction of samples followed by HPLC analysis of the 12 soy isoflavone forms has been done in methanol, ethanol, acetone, and acetonitrile with water and/or dilute acid. Each matrix requires different proportions of solvents to maximize extraction yield. Since malonyl forms decarboxylate in solution, HPLC must be performed immediately, or correction factors must be calculated to compensate for redistribution of the forms.[12] The authors have reported that 80% acetonitrile with 0.1 *N* HCl yields optimum extraction of all forms in most foods.[15] Extraction with acetonitrile without acid works very well for most foods. Methanol does not extract the acetylglucosides very efficiently.[24]

B. Data Presentation

Isoflavone content of foods or other products can be presented in two ways by manufacturers. For appropriate standardization, the concentrations reported in foods must clearly identify whether the mass of each glucoside form or the moles of each glucoside form are reported and used in calculating totals for each isoflavone. Ideally, the molar concentration of isoflavones should be used. However, since many soy products are used by those not familiar with scientific units, mass concentrations units (mg/g and μg/g) are used on some retail soy products. Therefore, it is recommended that total isoflavone contents be adjusted for the molecular weight differences of the glycoside moiety prior to addition.[8] Simple addition of all forms will overestimate true isoflavone dose by almost a factor of 2. Literature citations are not always clear on this point, especially reports of "conjugated" isoflavones.[11,16,22,25] Soy isoflavones vary in soy foods according to the soybean variety, level of processing, and type of processing. Ranges of isoflavone concentrations are known but exact levels for a particular product should be measured by the investigator or supplier.

II. SOY FOODS

Soy ingredients are the major source of isoflavones used in a variety of human clinical and in animal studies to determine the mechanism(s) for the health protective effects of isoflavones. Unfortunately, some clinicians erroneously believe that all soy protein sources are equal with respect to isoflavone content and use it as casein is used in feeding studies. However, isoflavone contents of soy ingredients and foods depend on a number of factors including variety of soybean and crop year and on the type of processing used to produce the ingredients.[26] Soy foods may be divided into four classes: (1) soy ingredients, (2) traditional soy foods, (3) second-generation soy foods, and (4) foods where soy is used as functional ingredient (unpublished data).[27] Soy ingredients include raw (or unprocessed) soybeans, soy flours (defatted and full-fat), soy concentrates, soy isolates, texturized vegetable soy protein (TVP), and hydrolyzed soy protein. Traditional soy foods include soymilks, tofu, tempeh, natto, miso, and other soy-based foods traditional to Asian cuisine. Second-generation soy foods include items such as soy-based burgers and hot dogs, chicken and bacon analogues, and soy cheeses. Foods where soy is used as functional ingredients include baked goods in which soy flour is added, soy-based infant formulas in which soy is used to replace milk proteins, and foods in which soy hydrolysates are added to replace monosodium glutamate. Isoflavone levels and distributions in representative groups of these soy foods vary widely (Table 4.2). Additional data can be found in the USDA–Iowa State University Isoflavone Database.

A. Processing Effects

The isoflavonoid moieties of isoflavone glucosides are not destroyed by most conventional food-processing operations. But the glucose residues are altered substantially during processing. Toasting soy flakes increases the concentrations of the acetyl glucosides at the expense of the β-glucosides.[24] Fermentation to produce tempeh, miso, and natto causes microbial hydrolysis of the glucosides to produce aglycones.[26,28] Minimal heat processing converts substantial amounts

TABLE 4.2
Isoflavone Content of Representative Soy Foods (μg/g wet weight)

	%	Glucosides			Malonylglucosides			Acetylglucosides			Aglycones			Total			
	M[a]	D[b]	G	Gl	D	G	Gl	D	G	Gl	Dein	Gein	Glein	Dein	Gein	Glein	Total
Soybeans[c]	8	270	418	92	1565	1684	192	4	27	0	27	25	7	986	1175	168	2329
Soy Isolate[d]	5	138	378	39	81	194	25	119	241	17	25	46	11	216	521	59	796
Soy Concentrate[e]																	
Ethanol washed	6	19	36	15	28	35	15	25	35	0	0	6	0	40	67	9	116
Water washed	3	88	260	29	223	523	43	45	112	21	25	40	12	217	539	66	822
TVP	5	493	696	179	787	905	127	898	844	146	41	51	19	1238	1439	286	2963
Soy germ	8	3221	5244	1196	575	265	994	1088 6	3336	5298	257	97	743	8552	2883	7696	19131
Kinako (toasted soy flour)[f]	5	582	657	95	58	47	0	1252	1240	0	52	65	37	1134	1210	94	2438
Soymilk																	
Aseptically processed[g]	89	68	88	13	29	20	3	0	10	0	2	2	0	59	74	10	143
Pasteurized[g]	91	23	34	5	39	44	5	0	5	0	3	4	1	37	51	7	95
Tofu A[h]	83	59	99	18	86	113	16	8	12	0	8	11	2	91	138	22	251
Tofu L[h]	86	105	143	29	143	40	7	0	17	0	7	9	2	86	129	24	239

Miso[i]	53	62	89	10	32	37	8	14	23	0	25	29	12	88	117	23	228
Tempeh[j]	61	93	206	14	64	111	8	49	46	0	85	103	9	201	316	22	538
Natto[k]	59	483	562	129	0	9	9	20	38	0	25	41	14	566	696	101	1363
Edamame	68	43	58	66	383	280	189	0	0	0	0	15	0	221	198	143	562
Chicken analogue[l] raw	74	30	78	8	5	6	0	17	35	0	5	7	3	35	79	9	123
Soy hot dog[m] raw	59	11	17	5	0	0	0	7	13	0	0	3	0	10	21	3	34
Soy burger[n] raw	63	12	25	6	24	42	7	18	29	0	8	13	4	37	66	12	115
Soy/beef burger[o] raw		63	2	4	0	3	4	0	0	5	0	0	1	0	3	8	0

[a] Moisture.

[b] D = daidzin; G = genistin; Gl = glycitin; Dein = daidzein; Gein = genistein; Glein = glycitein; Total = moles of isoflavone × molecular weight of isoflavone.

[c] 1997 crop average.

[d] Protein Technologies, Supro 675.

[e] Archer Daniels Midland, Co.

[f] Toasted soy flour, purchased in Japan.

[g] Aseptically processed soy milk, Original Eden Soy Beverage; pasteurized soy milk, White Wave Silk Dairyless Soy Beverage.

[h] Tofu A = Azumaya, extrafirm, composite; L = Mori-nu, firm, silken, 6 city composite.

[i] Miso A = shiro (white), 6 bag composite from supplier.

[j] Tempeh, raw, 5 city composite, White Wave.

[k] Sumibi natto, purchased in Japan.

[l] Chicken = Worthington Foods FriChik, 5 city, 2 can/city composite.

[m] Loma Linda meatless franks, 5 city, 2 can/city composite.

[n] Harvest burger.

[o] School lunch USDA Commodity patty, 6-patty composite.

of the malonylglucoside to the β-glucosides in an equimolar distribution.[21,26] Additionally, although isoflavones are not destroyed by food processing, isoflavones can be lost in discarded fractions during soy food processing, just as soy ingredient manufacturing ethanol extraction of soy flours to produce soy concentrate causes isoflavone loss. Examples of these losses of isoflavones are when soy whey is discarded in tofu production and when dehulled soybeans are boiled during the first step in tempeh manufacture and isoflavones leach into boiling water.[26]

B. Soy Ingredients

Soy ingredients contain the highest concentrations of isoflavones. Soybeans contain variable amounts of isoflavones, depending on variety, crop growing year, and location, and concentration can vary by a factor of 5.[29] Among the soy ingredients, defatted soy flours contain the highest concentrations of isoflavones. The isoflavones tend to associate with the protein fraction in soybeans. There are no detectable isoflavones in soybean oils. Full-fat soy flours have lower isoflavone levels than do defatted soy flours due to the dilution of full-fat flours by the lipids. Full-fat flours are a limited section of the commercial marketplace due to their instability from rapid lipid oxidation. Soy isolates are the alkaline-soluble, acid-insoluble protein fractions produced from defatted soy flours to yield approximately 90% protein. The aqueous processing steps result in losses of isoflavones in discarded fractions. Soy concentrates are prepared from defatted soy flours by either aqueous or alcoholic extraction. The type of extraction solvent has a profound effect on isoflavone content due to the solubility of isoflavones in alcohols. TVPs are prepared by extrusion from defatted soy flours, concentrates, or isolates. Mahungu and colleagues[30] reported conversion of malonylglucosides to β-glucosides during extrusion of corn/soy blends that was similar to the conversion occurring during TVP production. But because the researchers extracted their samples with 80% methanol, the amounts of acetylglucoside forms were greatly underestimated. Soy hydrolysates are produced by enzymatic hydrolysis of soy proteins. The isoflavone levels of the hydrolysates depend on the isoflavone concentrations in the starting material, and are therefore highly variable. Two representative hydrolysate isoflavone levels are presented in Table 4.3. The resulting isoflavone contents of soy ingredients will reflect the concentrations of the starting materials, but the processing history may be unknown by the user of these products.

C. Traditional Soy Foods

Traditional soy foods include soy milk, tofu, miso, tempeh, natto, soy sauce, and kinako and are familiar to Asian populations and to many Western consumers. Except for soy sauce, these foods are good sources of isoflavones with a long history of use in East Asia. The level of isoflavones in these products depends on the isoflavone concentration of the starting soybeans. Kinako has the highest soy isoflavone content because it is essentially toasted soy flour. Soy milk contains isoflavone concentrations similar to the starting soybeans on a dry weight basis. The percent solids in the soy milk manufactured affects total isoflavone content on a per serving basis. Tofus typically contain one half to one third the isoflavone mass of the soy milk used in their manufacture.[21,25,26] Miso production does not result in the loss of isoflavones but in an equimolar redistribution of the forms with conversions of the β-glucosides to aglycones. All the malonylglucosides are converted to the β-glucosides in the initial autoclaving/cooking step.[28] Natto isoflavone content is similar to starting soybean isoflavone mass. During the 18-h fermentation, a proportion of the glucosides is converted to aglycones (unpublished data). Soy sauce contains very low concentrations of isoflavones.[26] This may be the result of losses due to catabolism of isoflavones by fermentation organisms during the long fermentation and aging process for soy sauce production. Roasted or fried soybeans known as "soynuts" usually have the highest isoflavone concentration among available food products. These are whole soybeans that have been hydrated and fried or roasted. The loss of water concentrates the isoflavones. The intense heat processing redistributes the isoflavone forms but does not

TABLE 4.3
Isoflavone Content of Soy Protein Hydrolysates (μg/g wet weight)

		Glucosides			Malonylglucoside			Acetylglucoside			Aglycon			Total			
	% M[a]	D[b]	G	Gl	D	G	Gl	D	G	Gl	Dein	Gein	Glein	Dein	Gein	Glein	Total
Soy Hydrolysate A	5	923	1092	171	81	57	0	225	206	0	56	40	15	785	869	124	1778
Soy Hydrolysate B	5	97	66	38	13	0	0	0	0	0	0	0	0	66	41	24	131

[a] Moisture.

[b] D = daidzin; G = genistin; Gl = glycitin; Dein = daidzein; Gein = genistein; Glein = glycitein; Total = moles of isoflavone × molecular weight of isoflavone.

result in any loss of total isoflavones from the starting soybeans. Edamame or green soybeans contain about 1/5 the isoflavone content of mature soybeans.[25] Processing soybeans for tempeh production results in 12% loss of isoflavones from intact soybeans to soaking water and 49% loss of isoflavones to cooking water from the dehulled soybeans.[21] Tempeh is a good source of isoflavones with commercial tempeh containing 500 μg/g or about one half the concentration found in the soybeans used.[26]

D. Reduced Fat Soy Foods

Recently, U.S. manufacturers have begun to market low- and no-fat versions of soymilk and tofu with highly variable isoflavone levels due to the different manufacturing techniques used to produce these reduced-fat products. Low- and no-fat soy milks can be produced by skimming the soy milk to reduce fat or adding soy isolate or soy concentrate to increase protein concentration and dilute fat concentration while maintaining the same percent solids content. These different processes for producing the soy milks result in very different isoflavone profiles. Low- and no-fat tofus are produced by adding soy isolate and/or soy concentrate to the products prior to coagulation to dilute fat and increase the percent solids and protein. In each product, the amount of added ingredient and the isoflavone content of the ingredient will alter the total isoflavone content of these reduced-fat products (Table 4.4).

E. Second Generation Soy Foods

Second-generation soy foods are defined as those foods where soy protein is used to replace muscle or dairy proteins. Typically, the isoflavone concentrations in these products are low because of the small amount of soy ingredient used in their manufacture or because the products were produced using soy protein ingredients (such as soy concentrate) with very low isoflavone levels (see Table 4.2). Soy burgers, soy/beef burger blends, soy cheeses, soy meat analogues, and soy hot dogs are the most widely available products.

F. Foods with Soy as Functional Ingredient

There are a number of foods produced with soy added for functionality purposes. One of the most predominant functionality uses of soy is addition of soy flour to baked goods. Most baked products allow up to 3% soy flour usage.[31] Soy flour may be used in bread manufacture to accelerate gluten formation in the kneading process. Soy flour is added to fried bread products to reduce fat uptake. These cooking processes do not result in loss of isoflavones but in interconversion of the malonyl-glucosides into β-glucosides.[12] One unique use of soy as a functional ingredient is soy-based infant formula. This product is currently produced with soy isolate as the replacement for dairy proteins. These products are quite consistent in isoflavone content across brands probably due to the source of commercial isolate used in the product formulation.[8] Another major use of soy for functionality purposes is use of soy hydrolysates for replacement of monosodium glutamate. Any product ingredient list containing soy hydrolysate or vegetable hydrolysate contains soy hydrolysate and the accompanying isoflavones. As noted above, soy hydrolysates are quite variable in their isoflavone content. Canned tuna with soy hydrolysate listed in the ingredients was analyzed for isoflavones, which were found in the tuna meat, but not in the water portion. Examples of the relatively low isoflavone levels in doughnuts and tuna are presented in Table 4.5.

G. Soy in Dietary Supplements

The newest source of isoflavones for consumers is in dietary supplements. There are four major sources used to produce supplements. The first source that was available on the market was soy germ or soy hypocotyls produced by Schouten Company. Soy germ contains isoflavones at ten

TABLE 4.4
Isoflavone Content of Low and No-Fat Tofus and Soy Milks (μg/g dry weight)

		Glucosides			Malonylglucosides			Acetylglucosides			Aglycones			Total			
	% M[a]	D[b]	G	Gl	D	G	Gl	D	G	Gl	Dein	Gein	Glein	Dein	Gein	Glein	Total
Soymilk, 1% fat[c]	91	452	591	70	18	28	0	0	0	0	16	0	23	302	407	45	754
Soymilk, no fat A[d]	87	43	102	15	10	21	0	0	0	0	12	18	0	43	92	10	145
Soymilk, no fat B[e]	91	86	119	22	12	20	0	0	0	0	8	9	0	67	94	14	175
Tofu, lite, firm[f]	90	663	1065	140	122	219	27	112	203	16	55	81	18	583	975	131	1689
Tofu, lite, extrafirm[g]	87	590	964	120	198	353	32	122	230	16	68	102	18	589	1019	120	1728

[a] Moisture.

[b] D = daidzin; G = genistin; Gl = glycitin; Dein = daidzein; Gein = genistein; Glein = glycitein; Total = moles of isoflavone × molecular weight of isoflavone.

[c] Westbrae lite, soybeans.

[d] Heath Valley Fat Free, soy protein.

[e] Westbrae Natural nonfat, soybeans and soy concentrate.

[f] Morinu lite lowfat, soybeans and soy isolate.

[g] Morinu lite lowfat, soybeans and soy isolate.

TABLE 4.5
Isoflavone Content of Foods with Soy Protein Added as Functional Ingredient (μg/g dry weight)

		Glucosides			Malonylglucosides			Acetylglucosides			Aglycones			Total			
	% M[a]	D[b]	G	Gl	D	G	Gl	D	G	Gl	Dein	Gein	Glein	Dein	Gein	Glein	Total
Donuts A[c]	17	15	23	13	35	41	0	0	25	0	0	0	0	27	50	8	85
Donuts B[d]	21	17	23	13	23	28	14	20	29	0	7	0	0	40	45	16	111
Pancake mix[e]	5	23	31	15	83	89	22	0	28	14	4	0	0	60	82	29	171
Tuna[f]	77	5	9	0	5	6	0	0	0	0	4	4	0	9	13	0	22

[a] Moisture.

[b] D = daidzin; G = genistin; Gl = glycitin; Dein = daidzein; Gein = genistein; Glein = glycitein; Total = moles of isoflavone × molecular weight of isoflavone.

[c] Hostess powdered, soy flour, 61 g/serving.

[d] Cake, Cub Foods, soy flour, 60 g/serving.

[e] Krusteaz buttermilk pancake mix, soy flour, 57 g/serving.

[f] Packed in water, hydrolyzed soy protein, 56 g/serving.

times the concentration found in soybeans (see Table 4.2). The distribution of the isoflavones is quite different as well. Daidzein is the predominant form with glycitein second highest and genistein the minor form with a ratio of 50:40:10. The next commercial source marketed was the extract produced when soy concentrate was produced by ethanol extraction. This product is marketed as "Novasoy." The isoflavone distribution is comparable to soybeans with genistein:daidzein:glycitein in a ratio of 50:40:10. The third commercial supplement source is from red clover. This product contains the clover isoflavones formononetin and biochanin A and the coumestan coumestrol. The fourth commercial source is kudzu extract. Kudzu is an Asiatic plant and plant introduction escapee in southeastern United States. These extracts contain daidzein and puerarin as the isoflavones. Supplement manufacturers utilize one or combinations of these ingredients in producing their products. There are also a number of minor products produced in China and the United States by fermentation of different plant materials containing isoflavones. There are currently more than 100 supplement options on the U.S. market. Isoflavone supplements are typically tenfold or more concentrated compared with any food source of isoflavones. Whereas soy foods containing isoflavones have been consumed for over a thousand years, soy supplement use is a relatively new phenomenon.

Isoflavones may have toxic effects when consumed in amounts well above those found in soyfoods. There has been concern about isoflavone levels consumed by infants as part of soy-based infant formulas. However, in the more than 50 years of soy-based infant formula use in the United States, there has been no documented problem associated with the soy phytoestrogens or isoflavones. Because isoflavones have weak estrogenic activity, at high enough doses, they would be expected to act as estrogens. Lee and colleagues[1] showed a small effect of an isoflavone extract to promote putative hepatic pre-neoplasia in rats fed 480 mg total isoflavones/kg diet. It would not be possible to consume enough intact soy foods to obtain such a great isoflavone dose (e.g., a dose of about 240 mg isoflavones per person per day). Only concentrated extracts of isoflavones found in some dietary supplements could come close to these levels. Lee and colleagues[1] estimate a twofold margin of safety for isoflavones, because 240 mg total isoflavones/kg was an effective anticarcinogenic dose in rats given diethylnitrosamine to initiate hepatocarcinogenesis and then fed phenobarbital to promote hepatocarcinogenesis, whereas 480 mg isoflavones/kg promoted preneoplasia even in the absence of phenobarbital. This is a relatively narrow margin of safety but comparable to that observed with certain nutrients, e.g., selenium which is cancer protective at 2 mg/kg diet and toxic at 5 to 6 mg/kg diet in animals.[32]

III. SOY ISOFLAVONE BIOAVAILABILITY

A major determinant of the safety and efficacy of isoflavones is their bioavailability. Bioavailability is the access of a compound to its sites of action. The solubility, metabolic fate of compounds due to endogenous and exogenous biotransformation, and interaction of the compounds with other dietary components all determine isoflavone bioavailability.[33]

A. Apparent Absorption of Isoflavones

Physicochemical interactions of the isoflavones with the gastrointestinal mucosa seem to depend partly on the relative hydrophobicity/hydrophilicity of the compounds. There is no evidence for facilitated or active transport of these compounds, and the aglycone molecular weights should permit their diffusion (see Table 4.1). Although isoflavones are predominately in glycosidic form in foods, isoflavone glycosides have not been detected in human blood plasma or urine.[34,35,36] Thus, either human intestinal or gut microfloral glycosidases must act before the isoflavones are absorbed. Isoflavone aglycones elute in the following order during reverse-phase HPLC: first daidzein, then glycitein, and later genistein.[27,34,37] Although genistein has three hydroxyl groups and daidzein only two, a hydrogen bond is created in the genistein structure, increasing its

hydrophobicity compared with daidzein. Consistent with its greater hydrophobicity, genistein seems to be retained in the human body to a greater extent than is daidzein. For example, men and women fed soy milk containing 13% more genistein than daidzein had apparent peak plasma genistein concentrations of about 50% greater than peak daidzein concentrations (at 6 h after dose). Genistein plasma concentrations were twofold greater than daidzein even at 24 h after dosing.[38] But genistein seems to be less well absorbed than is daidzein, because the relative proportion of ingested daidzein excreted in urine is two- to threefold greater than the proportion of ingested genistein excreted in urine. This difference in urinary disposition may be due to greater gut microbial degradation of genistein than of daidzein[35] after the initial biliary excretion of the compounds,[39] rather than to the solubility differences of the compounds. On the other hand, glycitein is more hydrophobic than is daidzein, according to its relative HPLC retention time. Glycitein is seemingly absorbed to a greater extent than is daidzein, as reflected in relative urinary excretion of ingested dose.[38] But these data also suggest that glycitein is more readily eliminated in urine than is daidzein (55% of the total dose of glycitein vs. 47% of the daidzein dose eliminated over 48 h after dosing, compared with 29% of the genistein dose eliminated in urine, all significantly different, $p < 0.05$). The relative retention of glycitein in blood plasma is similar to that of daidzein in men but significantly less than daidzein in women,[38] for unknown reasons. Glycitein degradation by gut microorganisms has not been studied, so whether this also explains the relative apparent absorption of glycitein compared with the other isoflavones remains to be seen. The determinants of the relative absorption and disposition of the isoflavones seem to be complex, and not totally explained by relative compound solubility.

B. Endogenous Biotransformation of Isoflavones

Isoflavones seem to be rapidly and predominantly glucuronidated in the gastrointestinal mucosa, if genistein can be considered to be a model for all the isoflavones.[39] Further glucuronidation occurs in the liver. It seems that biliary excretion is a major fate of the isoflavones, with more than 70% of a dose recovered in bile within 4 h after dosing in rats. Thus, even if isoflavones are initially well absorbed, a maximum of about 25% of an oral genistein dose would be eliminated in urine. This is similar to the percentage of genistein dose recovered in urine as reported in human studies (17%,[40] 28%,[38]). Genistein may be excreted to a greater extent in bile than is daidzein or glycitein by inference from rats[39] and from the relative urinary recoveries reported for these compounds.[38] Because isoflavones seem to be conjugated before elimination, the relative extent of retention of the compounds in the body may depend on relative solubilities and other properties of isoflavone metabolites that have not yet been well characterized. The 7-*O*-glucuronides of daidzein and genistein follow the retention pattern of the parent isoflavones (genistein glucuronide the more hydrophobic of the two). Thus, greater biliary excretion of genistein than daidzein would be expected because the biliary route would be somewhat more hydrophobic than the urinary route of excretion.

Peterson and colleagues[41] identified sulfate and hydroxylated and methylated metabolites of genistein in breast cancer cell lines. The production of hydroxylated and methylated genistein metabolites correlated with inhibition of cancer cell proliferation, but the sulfates were not associated with antiproliferative effects of genistein, suggesting that some types of metabolism of the isoflavones may be crucial for their action. Zhang and colleagues[37] showed that genistein and daidzein glucuronides were about an order of magnitude less estrogenic than their respective isoflavone aglycones, as measured by *in vitro* binding with mouse uterine cytosolic estrogen receptor. The glucuronides also possessed modest ability to enhance human natural killer cell activity *in vitro*, comparable in magnitude with genistein, but effective and nontoxic over a wider concentration range than genistein. As little as 0.1 μM to 0.5 μM isoflavone or isoflavone glucuronide concentrations affected NK activity, which constitute readily achievable plasma levels after consumption of soy foods.[34,35,37]

In vitro studies of isoflavone effects might be misleading unless isoflavone metabolism and metabolites are considered. Isoflavone metabolism seemingly has importance to bioavailability and efficacy of these compounds. Characterization of physiologically relevant metabolite levels and patterns is likely to be necessary to determine the mechanisms of isoflavone action.

C. Microbial Biotransformation of Isoflavones

Isoflavone activity may also be altered by microbial biotransformation. Equol, another estrogenic isoflavone, is produced from daidzein in some individuals after a few days of repeated exposure to soybean ingredients.[42] One of six men fed soy for several weeks was an equol producer,[43] but four of six women fed soy for several weeks were equol producers.[44] In a study of 30 men and 30 women fed soy protein for 4 days, 35% of the subjects excreted equol after 3 days of soy feeding, with no significant gender difference.[45] After feeding soy flour to 12 subjects for 3 days, equol production was inversely related to the production of *O*-desmethylangolensin (ODMA), another daidzein metabolite produced during gut fermentation.[46] Kelly and colleagues[46] also identified several other urinary isoflavone metabolites likely to have been derived from gut microbial fermentation, including 6-hydroxy-ODMA, dehydro-ODMA, dihydrogenistein, and two isomers of tetrahydrodaidzein, and confirmed the presence of dihydrodaidzein. The health significance of these metabolites is unclear. But in a breast cancer case-control study in Shanghai, patients with breast cancer had lower urinary levels of the two major isoflavone metabolites, ODMA and equol, as well as of the three major soy aglycones, in comparison with matched controls.[47] It seems likely that any isoflavone metabolite present in readily measurable amounts could serve as a qualitative biomarker of soy intake. Estrogenic isoflavone metabolites, e.g., equol, might be especially useful in clarifying the health significance of soy, although the isoflavones seem to exert other effects in addition to their relatively weak estrogenicity, Some isoflavones are antiangiogenesis agents[48] and tyrosine kinase inhibitors.[49] But quantifying soy intake and health effects by examining the isoflavones and their metabolites is complicated by the gut microbial diversity that seems to be at least partly responsible for profound interindividual differences in isoflavone metabolism.

Interindividual variation in isoflavone metabolism and degradation by gut microorganisms seems to follow certain patterns. In a study of bioavailability of isoflavones from three soy milk meals over a day, two women consistently showed 10- to 20-fold greater fecal excretion of isoflavones in feces compared with the other five women. This paralleled two- to threefold greater urinary and plasma levels of isoflavones in the "high excretors" compared with the other five subjects.[35] *In vitro* isoflavone degradation by human fecal samples was shown to occur rapidly for both genistein and daidzein (degradation half-lives of 3.3 and 7.5 h, respectively). The more rapid degradation of genistein than of daidzein may account for the seemingly lesser absorption of genistein than of daidzein. This is reflected in relative urinary excretion as proportion of dose of these compounds (urinary excretion of daidzein two- to threefold greater than that of genistein) observed in numerous studies.[34,35,37–39] Rat intestinal bacteria degrade flavonoids with a 5-OH group such as genistein, to a much greater extent than structures lacking the 5-OH, such as daidzein.[50] Human gut microorganisms seem to possess similar ability based on relative apparent absorption of the compounds and their relative fecal degradation.[35]

A study of *in vitro* human fecal isoflavone degradation with 20 men and women showed that subjects sorted into three distinct degradation phenotypes, with degradation rate constants for genistein of 0.023 (high degrader, $n = 5$), 0.163 (moderate degrader, $n = 10$), and 0.299 (low degrader, $n = 5$).[51] These phenotypes seemed to be relatively constant when reexamined 10 months later with degradation rate constants of 0.049 (high degrader, $n = 5$), 0.233 (moderate, $n = 4$), and 0.400 (high, $n = 5$). Of the 14 subjects who remained in the study after 10 months, 12 maintained their initial isoflavone degradation phenotype (data not shown). When 8 men of varying degradation phenotypes were fed soymilk, plasma isoflavone contents correlated negatively and significantly ($p < 0.05$) with degradation rate constant, $r = -0.88$ for daidzein and $r = -0.74$ for daidzein.[52] These

data suggest that part of the interindividual variability in isoflavone disposition might be accounted for by variation in gut microbial isoflavone degradation rate. When only moderate isoflavone degraders were chosen for an isoflavone bioavailability study, the intersubject variation in urinary excretion of isoflavones was five- to eightfold. In a study of 14 subjects fed different doses of soy protein for 9-day intervals, within-treatment intersubject variation was 12- to 15-fold.[53] This suggests that selection for isoflavone degradation phenotype might lessen intersubject variation, but this needs to be studied in well-controlled experiments.

Isoflavone degrading microorganisms remain to be identified. Some *Clostridia* strains may cleave the C-ring of flavonoids, including isoflavones.[54] *Clostridia* are absent from the gastrointestinal tracts of some individuals and may be introduced during meat consumption.[55] Isoflavones can be broken down into other compounds that possess biological activity, especially monophenolics. For example, *p*-ethylphenol is a nonestrogenic metabolite of genistein identified in ruminants.[56] Methyl-*p*-hydroxyphenyllactate is a flavonoid metabolite that blocks nuclear estrogen receptor binding and inhibits the growth of MCF-7 breast cancer cells *in vitro*.[57] Characterization of their metabolism by gut microorganisms may yield insights into the mechanisms of action of isoflavones.

D. Dietary Interactions as Determinants of Isoflavone Bioavailability

Isoflavone bioavailability may also depend upon interactions between isoflavones and other dietary components. Isoflavones are associated with soy protein. The matrix for isoflavones within soybean foods and ingredients does not seem to have much impact on isoflavone bioavailability, although this has not been studied extensively. Isoflavone disposition from several soy foods was compared in a study in which single meals of tofu, tempeh, cooked soybeans, or TVP were fed to women in a randomized crossover design.[58] Urinary recoveries of the isoflavones over 24 h did not differ with soy food form (total recovery averaged 46% of ingested daidzein and 15% of ingested genistein among all soy foods), nor did plasma isoflavones. Women showed similar urinary isoflavone disposition whether they were fed tofu (high in malonyl glycosides) or TVP (high in acetyl glycosides).[59] These two studies together suggest that isoflavone aglycones, glycosides, and glycosides with different side chains are similar in bioavailability. But in a study feeding soybean pieces or tempeh to men for 9 days, tempeh isoflavones seemed to be twice as well absorbed as were isoflavones from soybean pieces, as reflected in urinary excretion.[60] Apparent isoflavone absorption was only 5 to 10% of the total dose in this study. In rats fed equimolar doses of the two isoflavone forms, plasma contents at 10 to 50 h after dosing and total urinary isoflavone excretion were similar, although genistein was more rapidly absorbed than was genistin.[61] It is probable that isoflavones from fermented soy foods such as tempeh, natto, or miso would be somewhat more rapidly absorbed, because the isoflavone aglycone content would be relatively increased in comparison with unfermented soy foods. Based on limited evidence, isoflavone glycosides and aglycones may be similar in overall bioavailability.

Other dietary factors seem to have little influence on isoflavone bioavailability. Choice of background diet did not affect isoflavone bioavailability when soy milk was fed for three meals on a single day to women in a randomized crossover design of three background diets. In one treatment, all subjects ate meals of the same composition at the same times. For a second treatment, subjects ate self-selected diets at the same times as the soy milk was fed. For the third treatment, subjects ate *ad libitum* for both diet choice and time but consumed the soy milk at the same times as for the other two treatments. No significant differences were noted among treatments with respect to plasma or urinary isoflavone contents or recovery as percentage of dose.[58] In 30 men and 30 women fed soy protein powder for 4 days, greater dietary fiber and total carbohydrate intake was associated with equol production in women but not in men, who consumed greater total dietary fiber than did women.[45] In women during a single day, 40 g of dietary fiber significantly suppressed total urinary genistein excretion by 20% after a soy milk meal compared with 15 g dietary fiber intake. The additional 25 g of dietary fiber was provided from wheat.[59] Thus, wheat fiber has only a modest effect on isoflavone bioavailability. The effects of other dietary factors on isoflavone bioavailability remain to be studied.

IV. HEALTH PROTECTIVE EFFECTS OF SOY ISOFLAVONES

Dietary soy protein may have an effect on blood cholesterol concentrations which was first reported in the 1940s by Meeker and Kesten.[62] However, the mechanism for soy protein's hypocholesterolemic effects in animals and humans is not known. Several different factors in soy proteins may be involved. Carroll and Kurowska[63] suggested that the amino acid pattern and peptide structure of soy protein may be the hypocholesterolemic factor. Setchell[64] suggested isoflavones as the active agent in soy. Potter and colleagues[65] reported that soy saponins and soy protein interact to alter plasma lipid concentrations.

A number of epidemiological studies have suggested that consumption of soybeans and soy foods is associated with lowered risks for several cancers, including breast, prostate, and colon,[66] cardiovascular diseases,[67,68,69,70] and bone loss.[71] The Food and Drug Administration (FDA) recently approved the Protein Technologies International, Inc. health claim petition for soy protein protecting against coronary heart disease.[69] There is good evidence that soy isoflavones benefit bone health by preventing bone loss for perimenopausal and postmenopausal women.

Lovati and colleagues[72] were among the first to suggest a role for the soy storage proteins having the ability to lower serum cholesterol. Sirtori and colleagues[73] pointed out that many of their reports on soy diets fed to very hypercholesterolemic children were devoid of isoflavones and still yielded a cholesterol reduction. Lovati and colleagues[72] showed that peptides from β-conglycinin, one of the soy storage proteins, upregulated hepatic low-density lipoprotein receptors in Hep G2 cells while glycinin peptides had no effect. Manzoni and colleagues[74] showed protein extracted from soybean mutants deficient in α′ peptide of β-conglycinin failed to upregulate the low-density lipoprotein receptor in a hepatic cancer cell line. Manzoni's soy preparations were very low in isoflavones while Lovati's were similar to soy isolates (unpublished data, Murphy, 1998). The authors have recently demonstrated that soy isolate without isoflavones reduces total serum cholesterol in hamsters compared with casein and to the same extent as intact soy isolate.[75] However, diets of casein plus daidzein, one of the three isoflavones, resulted in the same extent of plasma cholesterol lowering as did diets containing soy proteins. Balmir and colleagues[76] reported data very similar to the authors' using rats and hamsters. Potter and colleagues[77] reported plasma cholesterol lowering in hamsters fed soy isolate (probably containing isoflavones) and soy concentrate (probably devoid of isoflavones). Tovar-Palacio and colleagues[78] reported that cholesterol lowering occurred with soy protein alone or with three increasing levels of isoflavones compared with casein controls in gerbils. They reported no difference between intact soy protein and soy protein diets with additional isoflavones. Similar to the authors' data, their data yielded a maximizing effect for soy in cholesterol lowering. Kirk and colleagues[79] reported that dietary soy proteins with isoflavones lowered plasma cholesterol concentrations in CB57BL/6 mice but not in LDL receptor deficient mice. Ni and colleagues[80] have suggested that both the protein components and the ethanol extractables contribute to antiatherogenic effect of soy protein isolate. A meta-analysis of 38 human soy feeding studies (27 with sufficient resolution power) suggested that soy protein diets can lower serum cholesterol concentrations in moderately hypercholesterolemic human subjects (total cholesterol = 200 to 240 mg/dl) by 10%.[70] The level of soy in the diet needed to cause this effect averaged 47 g/day. Anderson and colleagues[70] did suggest that modest cholesterol lowering would probably be observed in subjects consuming 30 g/day. This dose is a level that can reasonably be incorporated into an American diet with available soy foods such as soy milk, tofu, tempeh, and misos. In the survey by Anderson and colleagues,[70] the soy protein source in diets was either isolated soy protein or TVP. It is possible to estimate with some confidence the isoflavone levels in isolate. However, TVP is an unknown since it may be produced from soy flours or isolates, both with isoflavones, or from concentrates, generally devoid of isoflavones. One cannot attribute all the hypocholesteremic effects of soy to isoflavones alone. The FDA has reached a similar conclusion in supporting the health claim petition by Protein Technologies, International, Inc. for soy protein, but not for the involvement of isoflavones, in contributing to improved cardiovascular

health for humans.[69] In view of the cited literature, it appears that soy proteins are playing some role in cholesterol regulation.

V. SUMMARY

Many plant constituents seem to have biological activities that affect cell function and therefore nutritional status and health. It is a very exciting and vital part of the food industry to determine how these components may be used in foods to enhance human health.

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5 Soy Protein, Soybean Isoflavones, and Bone Health: A Review of the Animal and Human Data

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I. INTRODUCTION

Osteoporosis, a worldwide problem of immense magnitude, may be defined as a metabolic bone disease with characteristic low bone mass and microarchitectural deterioration of bone tissue, which may lead to enhanced bone fracture risk.[1] In 1990, there were 1.66 million hip fractures worldwide.[2] However, as the world population ages, osteoporosis will result in marked increases in morbidity and mortality within the next 50 years, especially in Asia, Latin America, the Middle East, and Africa.[3]

Osteoporosis is the most prevalent metabolic bone disease in the United States and other developed countries.[4] It afflicts an estimated 25 million people in the United States and accounts for 1.5 million new fractures each year, and it is estimated that osteoporosis will cost society an

0-8493-8734-5/01/$0.00+$.50
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estimated $60 billion by the year 2020.[5] The World Health Organization (WHO) has proposed guidelines categorizing bone loss based on bone mass measurement at any skeletal site in Caucasian women. For example, a normal bone mineral density (BMD) is within one standard deviation (SD) of a "young normal" adult (T-score above –1 SD). Osteopenia includes BMD between 1 and 2.5 SD and osteoporosis includes BMD 2.5 SD or more below that of a "young normal" adult (T-score at or below –2.5 SD). Women in this latter group who experience one or more previous fractures are classified as having severe or established osteoporosis based on these WHO categories. It has been estimated that 54% of the postmenopausal Caucasian women in the United States have osteopenia and 30% have osteoporosis.[6]

Because of the important influence of early lifestyle factors on maintaining healthy bones later in life, osteoporosis is often viewed as a pediatric disease with a geriatric outcome. However, recent research has demonstrated that in older persons, both dietary and lifestyle approaches not only retard age-related bone loss but actually increase BMD.[7–10] Thus, dietary interventions aimed at the latter stages of life should hold promise for reducing fracture rates.

Dietary factors considered to influence bone either directly or indirectly include protein, alcohol, caffeine, calcium, phosphorus, magnesium, sodium, potassium, zinc, fluoride, boron, vitamin K, vitamin D, and vitamin A intake. Although there is some controversy about the extent to which calcium influences bone health, the Food and Nutrition Board, Institute of Medicine, of the United States recently substantially increased dietary calcium recommendations.[11] Surveys indicate that few people in the United States consume the recommended calcium intake.[12]

II. ESTROGEN AND THE GENDER GAP

Approximately twice as many women as men develop osteopenia with fracture rates also being twice as high in women.[13] Furthermore, on average, men develop fractures approximately 5 years later than women.[14] The longer life span of women further increases their osteoporosis burden.

The critical role that estrogen plays in bone health is evidenced by several observations, perhaps the most important of which is the marked loss in bone mass during the years immediately prior to and following menopause. At menopause, women undergo an accelerated rate of bone loss that is most apparent over the subsequent decade and accounts for cancellous bone losses of 20 to 30% and cortical bone losses of 5 to 10%.[15] Further support for the role of estrogen in bone health is the ability of conjugated equine estrogens alone or in combination with progestins to prevent bone loss at the hip and spine[16] and to reduce hip fracture rates.[17] Estrogen inhibits bone resorption by preventing osteoclast activity, but the precise mechanism is not known. Of interest is the fact that both estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ) are present in bone cells.[18–20]

Despite the potential value of estrogen replacement in reducing the burden of osteoporosis, the public health impact of estrogen therapy is unclear because so few women continue to take estrogen for decades.[21] Surveys indicate that two thirds of women discontinue treatment within 5 years of initiation due to undesirable side effects.[22,23] Importantly, research indicates that treatment for less than 10 years, provided around the onset of menopause but then discontinued, has little if any effect on the incidence of fractures at age 70.[24] If therapy is discontinued, bone loss is similar to that which occurs immediately after menopause.[25] Due to noncompliance and thus loss of efficacy, researching alternatives to steroid hormone therapy is warranted. Soyfoods and soybean isoflavones are increasingly being viewed as one such alternative.

III. EMERGING INTEREST IN SOYFOODS IN RELATION TO BONE HEALTH

For much of this century nutritional interest in soyfoods was limited to their ability to provide high-quality protein. However, within the past decade, soyfoods and soybean constituents have

been widely investigated for their role in chronic disease prevention.[26,27] Three international symposia (1994, 1996, and 1999) have been convened on this subject.[28–30] Clearly, early enthusiasm for this area of research was fueled in part by the lower rates of heart disease and mortality due to breast and prostate cancer in Asia.[31,32] Numerous *in vitro*, animal, and clinical studies now provide a solid basis for continued investigation. Another potential role for soy may be that of maintaining and improving bone health.

Three observations provided the basis for initial speculation that soy might contribute to bone health. These are (1) the estrogenic properties of soybean isoflavones,[33,34] (2) the effectiveness of the synthetic isoflavone, ipriflavone, in reducing bone loss in perimenopausal and postmenopausal women,[35–37] and (3) the low hip fracture rates in Asian countries.[38,39] In addition, several researchers have found that soy protein, when substituted for animal protein, decreases urinary calcium excretion. A growing number of animal studies have now investigated the effects of both soy protein and isolated isoflavones on BMD. There have also been a limited number of clinical and epidemiological investigations. Overall, the evidence suggesting that soy protein and isoflavones improve bone health is promising, but still speculative. The goal of this chapter is to summarize this research as well as to provide general background information on isoflavones.

IV. PRIMER ON TRADITIONAL AND WESTERN SOYFOODS

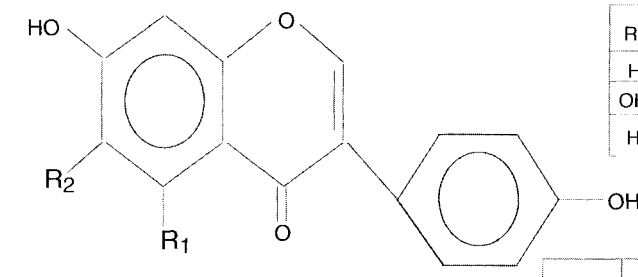
Traditional soyfoods such as tofu, miso, and tempeh have been consumed in Asian countries for centuries and continue to be common dietary staples in China, Japan, and Indonesia. It has been estimated, for example, that approximately 10% of the total per capita protein intake in Japan is derived from soy, or about 6 to 9 g of soy protein per day.[40,41] Soy consumption occurs in Japan primarily in the form of tofu, followed by the fermented soybean product miso.[41,42] Not unexpectedly, soy consumption in the West is quite low. Recent surveys suggest that less than 10% of U.S. Americans consume any traditional soyfoods whatsoever.[43–45] However, many Americans unintentionally consume small amounts of soy protein, no more than 5 g/day, in the form of processed foods to which soy protein has been added primarily for functional purposes (i.e., antioxidant effects, bleaching, and moisture retention).[46]

V. ISOFLAVONES

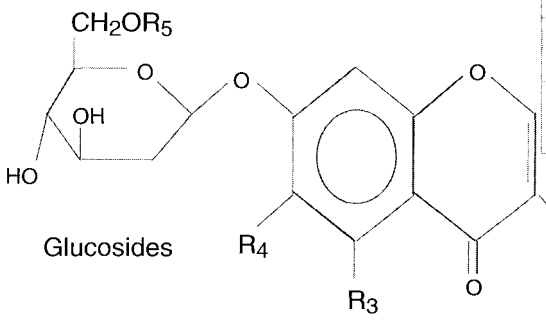
Nutritionally relevant amounts of isoflavones are found only in soybeans and soyfoods. Isoflavone content varies among soybean varieties although average concentrations are approximately 1 to 4 mg/g.[47,48] The isoflavone content of soybeans is markedly affected by growing conditions.[47–50]

Isoflavones are a subclass of the more ubiquitous flavonoids and have an extremely limited distribution in nature. The basic structural feature of flavonoid compounds is the flavone nucleus which comprises two benzene rings (A and B) linked through a heterocyclic pyrane C-ring (Figure 5.1). The position of the benzenoid B-ring divides the flavonoid class into flavonoids (2-position) and isoflavonoids (3-position). The primary isoflavones in soybeans are genistein (4′-5, 7-trihydroxyisoflavone) and daidzein (4′,7-dihydroxyisoflavone) and their respective β-glycosides, genistin and daidzin (sugars are attached at the 7-position of the A-ring). Typically, there is somewhat more genist(e)in than daidz(e)in in soybeans and soyfoods.[51–56] There are also small amounts of a third isoflavone in soybeans, glycitein (7,4′-dihydroxy-6-methoxyisoflavone) and its glycoside, glycitin.

In total, there are 12 different soybean isoflavone isomers; in addition to the 6 described above, each of the isoflavone glycosides can have an acetyl or malonyl group attached at carbon 6 of the A-ring (Figure 5.1). In soybeans, and in nonfermented soyfoods, isoflavones are present as β-glucosides, esterified with malonic or acetic acid.[57] In fermented soy products, due to bacterial hydrolysis, isoflavones are largely unconjugated. Dry heat, such as occurs in the toasting of soy



R1	R2	Compound	Molecular wt.
H	H	Daidzein	254
OH	H	Genistein	270
H	OCH_3	Glycitein	286



R3	R4	R5	Compound
H	H	H	Daidzin
OH	H	H	Genistin
H	OCH_3	H	Glycitin
H	H	$COCH_3$	Acetyldaidzin
OH	H	$COCH_3$	Acetylgenistin
H	OCH_3	$COCH_3$	Acetylglycitin
H	H	$COCH_2COOH$	Malonyldaidzin
OH	H	$COCH_2COOH$	Malonylgenistin
H	OCH_3	$COCH_2COOH$	Malonylglycitin

FIGURE 5.1 Chemical structures of the 12 isoflavone isomers.

flour, results in decarboxylation of the malonate group to acetate, forming 6′-*O*-β-acetyl glucoside conjugates, whereas hot water extraction results in complete loss of the malonyl group producing simple β-glucosides such as exists in soy milk and tofu.[57] Isoflavones are quite heat stable. Baking or frying does not alter total isoflavone content but increases the β-glucoside conjugates at the expense of 6′*O*-malonyl glucosides.[57]

A. Isoflavone Bioavailability and Serum Levels

Within the past few years, several groups have reported serum isoflavone levels in subjects after consuming soyfoods rich in isoflavones. Xu and colleagues[58] showed that in young adult women the absorption of a single dose of isoflavones from soy milk consumed in a controlled liquid diet was dose-dependent. In response to 0.7, 1.3, and 2.0 mg of genistein/kg body weight (bw), plasma concentrations 6.5 h after dosing were 1.53, 2.29, and 4.39 μmol/l, respectively. In a follow-up study by this group in which multiple doses of isoflavones were administered, plasma genistein and daidzein levels reached 6.00 μmol/l.[59] Finally, King and Bursill[60] found that in six healthy men plasma genistein levels reached a maximum value of 4.09 μmol/l in response to 3.6 μmol (approximately 1 mg) genistein/kg bw.[60]

Serum isoflavone concentrations in free-living Asians do not appear to be as high as those reported in subjects fed soyfoods in clinical studies. Adlercreutz and colleagues[61] reported that plasma genistein concentrations were only 276 nmol/l in free-living Japanese men ($N = 14$) although values twice this high were reported by Griffiths and colleagues.[62] The lower level in free-living Asians is not surprising given that typical Asian isoflavone intake is approximately 20 to 40 mg/day.[41,63] This is much less than that which is commonly ingested by subjects in studies examining isoflavone absorption and metabolism.

B. Isoflavone Physiology

Isoflavones have a strikingly similar chemical structure to mammalian estrogens. Therefore, it is not surprising that isoflavones bind to estrogen receptors and affect estrogen-regulated gene products.[33,34] Depending upon the assay employed, isoflavones are quite weak, possessing between 1×10^{-4} and 1×10^{-2} the activity of 17β-estradiol on a molar basis.[33,34,64] Importantly, Kuiper and colleagues[65,66] found that genistein binds with from 5 to 20 times more affinity to ERβ than ERα and that it has a binding affinity for both receptors that exceeds that of daidzein.[65,66] The stronger affinity of genistein for ERβ may be particularly important because, as noted previously, ERβ has been identified in bone tissue.[18–20] Thus, it may be necessary to adjust our thinking about the relative *in vivo* potency of isoflavones compared with endogenous estrogens. Furthermore, the greater binding affinity of isoflavones to ERβ than ERα suggests that isoflavones may be tissue selective in their effects, since some tissues contain predominantly one form of the receptor or the other. Interestingly, although research on glycitein is extremely limited, the *in vivo* estrogenic activity of this isoflavone based on the ability to stimulate uterine weight in mice is greater than that of genistein, even though it has a lower binding affinity for ERα.[67]

Although isoflavones are weak estrogens, the physiological effects of isoflavones, especially genistein, are likely only partially related to direct interaction with or binding to estrogen receptors. This is evidenced by the finding that genistein inhibits the growth of a wide range of both hormone dependent and independent cancer cells *in vitro*[26,68,69] — a result thought to be due to the ability of genistein to influence signal transduction. *In vitro*, genistein inhibits tyrosine-specific protein kinases,[70] mitogen activated kinases (MAP),[71] DNA topoisomerase II,[72] epidermal growth factor–induced phosphatidylinositol turnover,[73] and S6 kinase activation.[74] In addition to these effects, Kim and colleagues[75] reported that genistein increases *in vitro* concentrations of transforming growth factor beta (TGFβ). This finding may be particularly important given the role that TGFβ may have in bone cell regulation.[76] Finally, isoflavones possess antioxidant activity[77–79] and some research indicates isoflavones stimulate the immune system both *in vitro*[67] and in animal models.[80]

VI. ANIMAL MODELS OF OSTEOPOROSIS USING ISOLATED ISOFLAVONES

Several studies published within the past few years have examined the effect of isolated isoflavones on BMD using the ovariectomized rodent model, which is accepted by the U.S. Food and Drug Administration as a model for studying osteoporosis.[81] With only one exception, these studies have found beneficial effects. The first study to examine this issue by Blair and colleagues[82] found that genistein (30 μmol/day), when fed to ovariectomized Sprague-Dawley rats for 30 days following surgery (removal of the ovaries), increased dry femoral bone ash weights ($P < 0.05$) by 12% relative to controls. Similar results were reported by Ishimi and colleagues[83] in that genistein administration (0.7 mg/day) for 4 weeks reduced ovariectomized-induced bone loss in the femur of female mice, although genistein was not as effective as 17β-estradiol (0.01 μg/day).

Fanti and colleagues[84] also found genistein to be protective in ovariectomized Sprague-Dawley rats but three different doses of genistein were used in their study. Rats were injected with 1, 5, or 25 mg/kg bw per day for a period of 21 days immediately following surgery. Whole-tibia BMD of rats injected with either the 5 or 25 mg/kg dose, but not the low dose, was significantly higher than animals given the vehicle only. The intermediate dose was slightly more beneficial than the higher dose, although there were no statistically significant differences between these two groups. Unlike the findings by Fanti and colleagues, Ishida and colleagues[85] found that daidzin retarded femoral bone loss in ovariectomized Sprague-Dawley rats in a dose-dependent manner. Rats were given 10, 25, or 50 mg daidzin/kg bw by gavage for 28 days beginning 7 days after surgery. The decreases in femoral density and bone strength, as assessed by yield force, ash weight, and calcium and phosphorus content experienced by ovariectomized rats, were largely prevented by the high dose of daidzin (50 mg/kg) or genistin (50 mg/kg), or by estrone (7.5 mg/kg/day).

There may be two explanations why Ishida and colleagues observed a dose–response effect over the range of 10 to 50 mg daidzin/kg bw, and that in the study by Fanti and colleagues[84] genistein at a level of 25 mg/kg was no more effective than 5 mg/kg. First and most obvious, daidzin may act differently than genistein, although Ishida and colleagues[85] did not find this to be the case. Second and more likely, given isoflavone absorption rates of less than 50%, the subcutaneously administered 25 mg/kg dose used by Fanti and colleagues may have exposed rats to greater isoflavone concentrations than the highest oral dose employed by Ishida and colleagues.[85] Thus, the highest dose used by Fanti and colleagues may have more than surpassed the concentration required to exert maximal effects.

In contrast to results from both Fanti and colleagues[84] and Ishida and colleagues,[85] Anderson and colleagues[86] observed a biphasic effect of genistein on bone tissue. They found that in ovariectomized lactating rats fed a low-calcium diet, dietary genistein at a level of 0.5 mg/day (based on rats weighing approximately 300 g, this dose was equivalent to 1.7 mg/kg bw) was as effective as conjugated estrogen (16 μg/day) in retaining cancellous tibiae tissue, whereas the higher doses of genistein (1.6 and 5 mg/day) were less effective than the low dose. However, it is unclear how the lactating model employed by Anderson and colleagues compares to the ovariectomized rat model.

The only study that did not find a favorable effect of isoflavones (25 μg/g diet) did find that both 17β-estradiol and the phytoestrogen coumestrol retarded bone loss in ovariectomized rats.[87] However, in this study, rats were fed isoflavones derived not from soy, but from red clover which contains mostly the methylated isoflavones formononetin and biochanin A, with only small amounts of genistein. The extent to which the methylated isoflavones differ from genistein and daidzein in their effects on bone is unclear, but it is thought that *in vivo* most formononetin and biochanin A are converted into daidzein and genistein, respectively.[88]

VII. ANIMAL STUDIES OF OSTEOPOROSIS USING SOY PROTEIN

More than 10 years ago, Kalu and colleagues[90] found that in comparison to casein, a soy-based diet delayed and also reduced the magnitude of age-related decreases in femoral BMD in Fischer 344 male rats. Whereas BMD rapidly began decreasing at about 24 months of age in the casein group, decreases in the soy group did not occur until 3 to 4 months later. Even more striking was the higher final BMD of the soy-fed animals. These results, while seemingly impressive, may not stem from a direct effect of soy protein on bone tissue because bone loss in a group of energy-restricted rats was reduced to a similar extent as in the soy-fed rats. Fischer male 344 rats are prone to develop renal problems and secondary hyperparathyroidism, which results in bone loss. Several studies have shown that soy protein has favorable effects on renal function relative to other proteins, including casein.[91,92] Thus, the effects of soy protein on bone tissue may have been mediated through effects on renal function.

In a shorter-term study, Arjmandi and colleagues[93] found that soy protein isolate (SPI, 20% by wt), when fed for 30 days immediately following surgery (ovariectomy or sham), retarded bone loss in the right femur and fourth lumbar vertebra compared to the casein-fed control. BMD of soy-fed animals at those sites was significantly higher than the ovariectomized control animals, and was equal to that of the rats given 17β-estradiol in the case of the fourth lumbar vertebrae, and in the case of the femur was intermediate between ovariectomized controls and estrogen-treated animals. Similarly, Harrison and colleagues[94] found that soy protein increased femur wet weight of ovariectomized Sprague-Dawley rats in comparison to the casein controls despite a delay in dietary treatments until 2 weeks after surgery. In addition, the ash weight and calcium content of the femur and tibia were significantly lower in the casein group in comparison to the sham group, whereas in both the soy and estrogen groups these values were significantly elevated in comparison to both the sham and casein groups.

This next study by Omi and colleagues[95] differs significantly in two ways from the previously described animal studies. (1) The soy protein source was soy milk, not soy protein isolate, and

(2) experimental diets did not begin until 29 days after ovariectomy. Nevertheless, they found that both the lumbar spine and tibia BMD (proximal metaphysis and diaphysis) were significantly elevated in rats fed soy milk for 28 days.[95] Mechanical bone strength and bone calcium and phosphorus content of the femur were also increased in the soy-fed animals, similar to the findings of Arjmandi and colleagues[93] and Harrision and colleagues.[94]

The four studies discussed above were not designed to determine whether isoflavones were the specific components of soy protein responsible for the effects on bone tissue. However, Arjmandi and colleagues,[96] using a similar design as described previously, found higher BMD in the group of rats fed an isoflavone-rich soy protein (soy+) which contained approximately 2 mg/g protein, but not in rats fed soy protein (soy–) from which the isoflavones had been largely extracted. Clearly, this suggests, but does not definitively show, that isoflavones are the components of soy exerting the bone-sparing effects.

Although protective effects of soy protein were observed in all four of the studies involving ovariectomized rats,[93–96] according to results by Arjmandi and colleagues, when soy feeding is delayed for some time after ovariectomy, the beneficial effects of soy protein are substantially diminished. When soy-containing diets were not fed to ovariectomized rats until 35 days after surgery, the BMD of the fourth lumbar vertebrae was significantly higher in the sham group than in the casein and soy groups (soy+ and soy–).[97] Although femoral BMD was significantly higher in the sham group compared with the control (casein) group, there were no statistically significant differences between the two soy groups and the sham and casein-fed groups. Thus, a delay in feeding reduced the effectiveness of soy protein to retard trabecular bone loss as evidenced by histomorphometric analyses, but still allowed for some attenuation of cortical bone loss as evidenced by femoral bone density.

However, an aspect of the experimental design other than the treatment delay may have influenced the results of this study. Bone measurements were not taken until 65 days after soy feeding began. Most other studies were ended 30 days after treatment.[93–96] Thus, the loss of effectiveness may stem from the longer observation period. In other words, the rather unimpressive findings may have resulted from protective effects diminishing over time, rather than from a delay in treatment per se. This notion gains some support from the study by Omi and colleagues,[95] in which they observed protective effects of soy protein despite a delay in dietary treatments until 29 days after ovariectomy.

Finally, in the only study involving orchidectomized male rats, Juma and colleagues[98] found that soy diets regardless of isoflavone content had no effect on femoral BMD, but nevertheless increased bone strength as assessed by yield force resistance. Thus, independent of effects on BMD, soy might reduce the risk of osteoporosis by improving bone architecture.

In contrast to the favorable effects observed in rats, two studies in monkeys (cynomologus macaques) failed to show any protective effects of soy protein. In one, neither soy– nor soy+ retarded bone loss in ovariectomized monkeys during the 23 months of the experimental period, although monkeys given Premarin actually gained bone mineral content at the spine and whole body.[99] In a second study, whereas 17β-estradiol completely prevented ovariectomized-induced increases in cortical bone turnover, feeding a diet containing an isoflavone-rich soy protein for 7 months had no effect.[100]

VIII. HUMAN STUDIES

A. Bone Turnover

Relative little human research on the effects of soy protein or products on bone health has been conducted, and, at the time of this writing, much of that research is available only in abstract form. Nevertheless, studies are somewhat consistent in showing that soy inhibits bone turnover in postmenopausal women. The first group to examine this issue, Murkies and colleagues,[101] conducted

a short-term study (12 weeks) in which the diets of postmenopausal women were supplemented with either wheat or soyflour (45 g/day, 52 mg isoflavones). Although the primary end point of this study was hot flashes, urinary hydroxyproline, a nonspecific marker of bone resorption, increased significantly in the women in the wheat group but not in the soy flour group, although there were no statistically significant differences between groups.

Three other studies also found that soy feeding was associated with a decrease in bone resorption. Postmenopausal women fed 40 g of soy protein (76 mg of isoflavones) per day for 12 weeks exhibited decreases in urinary *d*-pyridinoline (8.1 vs. 7.3 n*M*/m*M* creatinine, $P < 0.05$) and decreases in urinary *N*-telopeptide (NTx) concentrations (69.4 vs. 53.3 n*M*/m*M* creatinine, $P < 0.001$), whereas there were no changes in these bone-specific markers in the placebo (casein) group.[102,103] In another study, soyfood (60 to 70 mg isoflavones/day) consumption was found to decrease urinary excretion of NTx by 13.9% ($P < 0.02$) and to increase serum osteocalcin by 10.2% ($p < 0.03$), suggesting an increase in osteoblastic activity.[104] However, there was no control group in this study. Interestingly though, there was a significant negative correlation between urinary NTx and serum isoflavone concentrations. Finally, Wong[105] reported that isoflavone supplementation (160 mg/day) decreased urinary concentrations of deoxypyrindinoline (Dpd) and increased serum concentrations of osteocalcin and bone-specific alkaline phosphatase activity, although differences were not statistically significant. However, since the magnitude of the changes were similar to those seen with estrogen administration, the lack of statistical significance was likely due to the very small sample size ($N = 6$).

The final study in postmenopausal women was by Washburn and colleagues,[106] who reported the effects of soy protein on alkaline phosphatase activity, a nonspecific marker for bone formation, in a double-blind randomized crossover study in which three different diets were fed to subjects for 6 weeks each. One of the diets was supplemented with 20 g carbohydrate and the other two were supplemented with 20 g soy protein (34 mg of isoflavones); one of these groups consumed soy protein once per day and the other twice per day. Alkaline phosphatase activity decreased significantly in women on either soy diet compared with the carbohydrate-supplemented diet ($P < 0.05$), suggesting that bone formation declined. However, since bone resorption markers were not examined, the significance of this finding, if any, is difficult to determine.

In contrast to the above studies, Alekel and colleagues[107] recently completed a trial in perimenopausal women ($N = 69$) randomly assigned (double-blind) to one of three treatment groups who did not exhibit any decline in bone resorption during the course of treatment. These women entered the study in four waves or cohorts with approximately equal presentation from each of the three treatments (isoflavone-rich soy protein isolate, or SPI+; isoflavone-deficient soy protein isolate, SPI–; and whey protein, control) in each cohort. Subjects consumed 40 g of protein (soy or whey) for 24 weeks. Repeated measures of ANCOVA indicated that both time ($P \leq 0.005$) and baseline value ($P \leq 0.0001$) were very significant, whereas treatment per se had no significant effect on either NTx ($P = 0.12$) or bone alkaline phosphatase ($P = 0.32$). Interestingly, cohort had a significant effect on NTx ($P = 0.0089$), but not phosphatase ($P = 0.56$), suggesting that cohort may reflect a seasonal effect on bone resorption.

B. Bone Mineral Density

Thus far, four studies, of which only two have been published in full, have examined the effect of soy consumption on BMD or bone mineral content (BMC). Three of the four studies found an attenuation of bone loss in perimenopausal or postmenopausal women. Dalais and colleagues[108] fed postmenopausal women 45 g of soy grits (flour)/day (52 mg of isoflavones) for 12 weeks and found that BMC significantly increased 5.2% ($P < 0.03$) although there was no change in BMD. However, not only is the magnitude of this increase surprising, but there were also increases in BMC, albeit not significant, in both the control group who were fed wheat flour and a group of women fed flaxseed. Clearly, these results need to be followed up to draw meaningful conclusions.

The next two studies provide support for the hypothesis that isoflavones are the component of soy responsible for the protective effects on bone. In the study by Alekel and colleagues[107] previously described, percentage change in lumbar spine BMD or BMC did not decline in the SPI+ or SPI– groups; however, significant loss occurred in the control group.[107] Absolute values for bone at baseline and post-treatment were not statistically different among the three groups. Results of ANCOVA indicated that treatment has a significant effect on percentage change in BMC (P = 0.021), but not on percentage change in BMD (P = 0.25). However, when various contributing factors were taken into account using multiple-regression analysis, SPI+ had a significant positive treatment effect on the percentage change in both BMD (5.6%, P = 0.023) and BMC (10.1%, P = 0.0032), while the other treatments had no effect. There was no effect of any treatment on bone sites other than the lumbar spine.

In agreement with the study by Alekel and colleagues are results from Potter and colleagues,[109] who found that in postmenopausal women consuming 40 g of soy protein/day (90 mg of isoflavones) for 6 months, there was a statistically significant increase in lumbar spine BMD, whereas there were decreases in BMD in the women fed 40 g of soy protein containing a lesser amount of isoflavones (56 mg) or 40 g of casein-based milk protein. However, as was also observed by Alekel and colleagues,[107] there were no differences among treatments at the other bone sites measured. The findings by Alekel and colleagues and Potter and colleagues, which suggest that isoflavones are responsible for the bone-sparing effects of soy protein, are consistent with those of Arjmandi and colleagues[96] in animals and Scheiber and colleagues[104] in women.

In contrast to the three previously cited studies, Gallagher and colleagues[110] found that soy protein, regardless of isoflavone content, had no effect on BMC. Over a 9-month period, early postmenopausal women were fed 40 g soy protein containing one of three levels of isoflavones: (1) little or no isoflavones, (2) 52 mg, or (3) 96 mg per day. There were no significant differences among the three groups in spinal, femoral neck, or trochanteric BMD during the intervention phase. Gallagher has commented that the lack of effect may have been because women in the study were 1 to 5 years postmenopausal (personal communication). During this period of time twice the dose of estrogen may be needed compared with that required by older women to reduce bone loss. Another consideration is that if components in soy other than isoflavones are responsible for the purported effects on bone, this study would not have been able to identify protective effects since soy protein was fed to each group.

IX. OSTEOPOROSIS IN ASIA

As noted at the onset, the low hip fracture rates in Asians have been cited as support for a beneficial effect of soy consumption on bone health.[38,39] However, several observations call this notion into question. First, two human studies cited above found that soy consumption was associated with favorable effects on spinal BMD but not on hip BMD.[107,109] Second, although the rate of hip fracture in Japan and China is low relative to the West, the Asian spinal fracture rate is similar to the West, and Asian lumbar spine BMD is similar to that of Caucasians.[111–118] Third, typical soy protein (6 to 9 g/day) and isoflavone intake (20 to 40 mg/day) in Asia is much lower than the amount of soy/isoflavones shown to be effective in the short-term clinical studies by Potter and colleagues,[109] Alekel and colleagues,[107] and to a lesser extent, Dalais and colleagues.[108]

It should be noted, however, that even though soy may not reduce hip fracture risk by increasing femoral BMD, soy may conceivably enhance femoral bone strength. As noted previously, Juma and colleagues[98] found that bone strength increased in male rats without a corresponding increase in BMD. Of course, whether soy exerts such an effect in humans is highly speculative. Also, although Asian isoflavone intake is less than that which has been associated with benefits in short-term clinical studies, it is certainly possible that lesser amounts of isoflavones consumed over the course of many years could have significant bone-sparing effects. In fact, as noted below, four epidemiological studies suggest this is the case.

Although many factors, including isoflavones, may contribute to the lower hip fracture rate in Asians, two in particular are especially noteworthy. One is the shorter hip axis length of Asians,[120–125] and the other is the lesser tendency of Asians to fall.[126,127] Since more than 90% of all hip fractures are due to trauma, these factors are likely to impact significantly on hip fracture rates.[128]

X. SOY INTAKE, BMD, AND FRACTURE RISK: EPIDEMIOLOGICAL INVESTIGATIONS

Three studies presented at the Third International Symposium on the Role of Soy in Preventing and Treating Chronic Disease (Washington, D.C., 1999)[129–131] and one recently presented at the American Society for Bone and Mineral Research[132] reported that soy intake and/or isoflavone excretion among Asians was correlated with higher BMD or decreases in bone resorption. South Korean researchers[129] found that among 60 postmenopausal women identified as high soy consumers, urinary isoflavone concentrations were negatively correlated with urinary deoxypyridinoline excretion. Similarly, Fukui and colleagues[130] reported that among middle-aged women living in Japan ($N = 39$, mean age 54.8 years) and elderly Japanese women living in Hawaii ($N = 48$, mean age 74.4 years) urinary isoflavone concentrations were positively and negatively correlated with BMD and bone resorption markers, respectively. In the United States, among 267 older Japanese women residing in the Seattle area, Rice and colleagues[13] reported that femoral neck BMD was significantly higher ($P < 0.03$) among high lifetime soy consumers than among low lifetime consumers (0.680 vs. 0.628 g/cm^2).[131] Finally, Ho and colleagues[132] found that isoflavone intake in young women from Hong Kong, 31 to 40 years of age, was positively correlated with spinal BMD over a 3-year period.

In addition to these findings published thus far only as abstracts, Fujiwara and colleagues[130] cited two Japanese case-control studies (published in Japanese) of hip fracture that reportedly found soyfood intake to be protective. One found that subjects with fractures consumed significantly less tofu than controls; however, this study involved only 40 pair-matched controls.[133] In the other study, natto (a fermented soybean product) consumption by prefecture as estimated by household expenditure was inversely related to hip fracture incidence.[134] However, study investigators attributed the protective effect of natto to its high vitamin K content. Because vitamin K is required for the carboxylation of osteocalcin, lower circulating concentrations of carboxylated osteocalcin and marginal intakes of vitamin K have been associated with fractures.[135,136] Furthermore, vitamin K supplementation significantly increased spinal BMD and decreased vertebral fracture risk over a 2-year period in a group of Japanese women with osteoporosis.[137]

In contrast to these favorable reports, several published studies have not found a relationship between bone health and soy intake. For example, Fujiwara and colleagues,[138] in a prospective study involving 1586 Japanese men (average age 58.2 years) and 2987 women (average age 58.6 years), noted that tofu intake was unrelated to the development of hip fractures. However, specific data on tofu intake and hip fracture risk were not presented and during the 12- to 14-year follow-up period only 55 incident hip fractures not due to traffic accidents were recorded. In agreement with these findings, Lacey and colleagues,[139] in a case-control study involving both premenopausal and postmenopausal Japanese women, found that soy intake was not significantly related to cortical bone mass (personal communication). Also, in a longitudinal study, although bone loss over time was lower in Asian men and women than in British subjects, tofu intake was not related to bone loss in Asians (personal communication).[116]

Clearly, there are little epidemiological data upon which to draw conclusions about the relationship between soy intake and fracture risk and the data that do exist are mixed. However, even if one assumes that soy favorably impacts bone, one might not expect epidemiological studies to show protective effects. Consider that in the case of calcium, the beneficial effects of which on bone health appear to be firmly established, calcium intake is frequently not found to be protective

in epidemiological studies.[140–142] Heaney[143] has discussed in detail why calcium, despite its beneficial effects, is not always identified as such in epidemiological investigations. Thus, the extent to which epidemiological data will provide insight into the effect of soy on bone health may be limited.

XI. PROTEIN INTAKE AND CALCIUM EXCRETION

In 1968, Wachman and Bernstein[144] proposed that a high protein intake, by increasing urinary calcium excretion, could lead to osteoporosis. Support for this hypothesis comes from many different types of studies. Most importantly, human feeding studies have demonstrated the hypercalciuric effect of isolated proteins, such as casein.[145–147]

In contrast to studies using isolated proteins, studies in which protein has been increased via the consumption of animal foods naturally high in protein have not always found a corresponding increase in urinary calcium excretion.[148] This is likely due to the phosphorus content that normally accompanies animal-based protein foods. Phosphorus mitigates the hypercalciuric effect of protein by increasing circulating parathyroid hormone, which in turn leads to a decrease in urinary calcium.[149,150] However, according to some research, phosphorus increases the calcium content of the digestive juice and therefore fecal calcium content to a similar magnitude as it reduces urinary loss, so that there is still an overall negative effect of protein on calcium balance.[150]

The importance of urinary calcium excretion on calcium balance has been demonstrated by several investigators.[151–153] In fact, Heaney[152] found in a series of balance studies involving over 500 women that urinary calcium excretion accounted for more than 50% of the variation in calcium balance, whereas calcium intake accounted for only about 10%. Because of the reported effects of protein on calcium excretion, the dietary calcium (mg) to protein (g) ratio has been suggested as a better indicator of calcium nutriture than calcium intake alone.[154] Accordingly, studies have found that the protein/calcium ratio is more predictive of fractures[155] and bone gain[156] than calcium intake. Every gram of protein ingested is believed to result in the excretion of approximately 1 mg of calcium.[151–153] This effect is not trivial since net calcium absorption at calcium levels typically consumed in Western countries may be no more than 10%.[151,157] It should be noted, however, that while excessive protein intake may increase osteoporosis risk, adequate protein intake is needed for good bone health.[158]

Although there may be several contributing factors, the hypercalciuric effect of protein is primarily attributed to the metabolism of the sulfur amino acids (SAAs) methionine and cysteine.[159,160] SAA metabolism results in the production of hydrogen ions, which requires buffering to maintain pH within the appropriate range. Because the skeletal system is the largest source of alkaline, bone is dissolved in response to the production of hydrogen ions.[161] This allows phosphate to be released as a buffering agent, which in turn leads to a rise in blood calcium concentrations and an increase in urinary calcium excretion.

The effect of SAAs on calcium excretion has been demonstrated experimentally. For example, Remer and Manz[160] have shown that as dietary protein increased from 49 to 95 to 120 g/day, urinary pH decreased from 6.7 to 6.0 to 5.5 with an increase in calcium excretion from 3.8 to 7.8 to 8.6 mEq/day. Furthermore, in this study, the consumption of 95 g of protein plus methionine supplementation equivalent to that found in the 120-g protein diet resulted in a similar urinary pH (5.4) and elevated calcium excretion to 9.6 mEq/day.

Increasing the alkalinity of the diet, such as by increasing potassium content, will have the opposite effect as increasing sulfur amino acid intake. For example, in the dietary alternatives health study (DASH) study,[162] maintaining protein at a constant level but more than doubling the potassium content of the diet from about 1700 mg/day to about 4000 mg/day by increasing vegetable intake resulted in a decrease in urinary calcium of 47 mg/day. Similarly, Kaneko and colleagues[163] found that in young Japanese women, supplementing soy protein with SAAs led to an increase in urinary calcium excretion whereas no increase was noted when potassium was added to this mix.

XII. SOY PROTEIN AND CALCIUM EXCRETION

On a mg/g protein basis, proteins differ markedly in their SAA content. Legumes, including soybeans, are relatively low in sulfur amino acids.[164] For example, soy protein contains 29.6 mg/100 g of protein, whereas milk and beef protein contain 33.8 and 34.2 mg/100 g of protein, respectively.[164] Thus, one would expect soy protein to be less hypercalciuric than animal protein. And, in fact, this has been shown to be the case in several human studies.[163,165–168]

Anderson and colleagues[165] compared the effects of whey and soy protein over a 24-h period. They found that the ratio of urinary calcium to creatinine increased by 45% 4 h following the consumption of a meal containing milk whey (2.8 g methionine/100 g) as the primary protein source, whereas in response to a meal containing a similar amount of protein from soy (1.3 g methionine/100 g) the ratio of calcium to creatinine increased by only 3%.[166] Whereas 20 h following the milk meal, the ratio of calcium to creatinine was 55.9% higher than baseline, this ratio was only 27.2% higher for the soy group.

The first longer-term study to demonstrate that soy protein is less hypercalciuric than animal protein was a 2-week (1-week/diet) feeding study involving four women and five men, aged 22 to 69 years. Subjects were fed approximately 80 g of protein derived primarily from either soybeans or chicken and similar amounts of calcium, phosphorus, magnesium, and sulfur.[166] Overall, in comparison to the baseline values, urinary total titratable acid increased only 4% on the soy diet but by 46% on the meat diet. Although there was considerable variation, urinary calcium averaged 4.23 and 5.07 mEq on the soy and meat diet, respectively.

Similar differences between meat and soy protein were noted in a slightly longer-term study by Breslau and colleagues.[167] Utilizing a crossover design, subjects (N = 15, 8 men, 7 women) consumed in random order each of three different diets for 12-day periods.[165] The protein (≈75 g/day), sodium (≈400 mg), calcium (≈400 mg), and phosphorus (≈1000 mg) contents of the diets were similar. However, one diet provided protein derived only from animal sources (eggs, beef, fish, chicken), one only from soy, and one from a combination of animal sources and soy. No differences in calcium absorption were noted while subjects were on the different diets but 24-h urinary calcium excretion was 150, 121, and 103 mg when subjects were on the animal protein, soy and animal protein, and soy protein diets, respectively. Calcium excretion on the animal protein diet was significantly different from the other two diets ($P < 0.02$).[165]

In a small study[168] involving young Korean adult women ($N = 6$), subjects were fed for 5 days first a meat-containing diet (≈71 g protein/day) and then a soy-containing diet (83 g protein/day). The calcium content of the diets was similar (≈525 mg/day) although the phosphorus content of the soy diet was higher than the meat diet (1033 vs. 769 mg). Daily urinary calcium excretion was 127 and 88 mg while subjects were on the meat and soy diets, respectively ($P < 0.025$). Fecal calcium excretion increased on the meat diet (467 vs. 284 mg) and overall calcium balance was significantly more negative (–65.4 vs. 155.3 mg; $P < 0.001$).[167]

Finally, Kaneko and colleagues[163] also found that adding meat protein (≈50 g) to a basal diet increased 24-h urinary calcium excretion in young women ($N = 7$) to a larger extent than a similar amount of soy protein (174 vs. 143 mg), although this difference was not statistically significant. However, there was no difference in overall calcium balance between the two diets because of a greater fecal calcium excretion on the soy diet. In a second experiment by these researchers with a similar design, urinary calcium excretion was again lower on the soy diet (154 vs. 136 mg/day) and overall calcium balance was markedly better (–34 vs. –114 mg of calcium).

In conclusion, although the data are limited, studies are consistent in showing a lower urinary calcium excretion in response to soy protein in comparison to a similar amount of animal protein. These findings assume added credibility because they are consistent with the recognized hypercalciuric effect of SAAs from protein. The clinical significance of substituting soyfoods for animal foods on calcium balance and hence bone health will, of course, depend upon the relative intake of soy. In general, it would appear to play only a minor role since daily per capita soy

protein intake, even among Asian people, averages only 6 to 9 g/day.[40,41] However, for individuals consuming two or more servings of soyfoods per day, which would provide as much as 20 g of soy protein, the favorable effects of soy protein on calcium excretion would likely be clinically quite relevant.

XIII. EFFECTS OF SOY ON BONE HEALTH: POSSIBLE MECHANISMS

A variety of mechanisms have been proposed for the favorable effects of soy protein/isoflavones on bone health. As noted previously, the effects of soy protein on renal function may have been responsible for the higher BMD in soy-fed rats in a long-term study.[89] Additionally, as just discussed, when substituted for animal protein, soy protein has been shown to result in lower urinary calcium excretion. However, these effects are likely not responsible for the favorable effects observed in clinical and epidemiological studies since both isoflavone-poor and isoflavone-rich soy protein would be expected to have similar effects on renal function and calcium excretion. In contrast, two human studies found that isoflavone-rich but not isoflavone-poor soy protein favorably affected BMD.[107,109] Furthermore, Scheiber and colleagues[104] found there was a significant negative correlation between urinary NTx and serum isoflavone concentrations. Thus, although the effects of soy protein on calcium excretion, and to a lesser extent renal function, may be clinically relevant, there is considerable evidence indicating that isoflavones have direct beneficial skeletal effects. In addition to the three human studies cited above, supporting evidence emerges from work in cells,[169–175] organ culture,[176–179] and animal models[82–86] in which isolated isoflavones have been employed.

The estrogenic effects of isoflavones are well established. Yet it is not clear that isoflavones exert their effects on bone by binding to estrogen receptors. Of interest are the results from several studies which found that soy protein or isolated isoflavones exerted favorable effects on BMD with either minimal or no increase in uterine weight in contrast to the markedly increased uterine weight in response to estrogen administration.[82–85,93] These data do not in any way preclude the possibility that isoflavones exert estrogenic effects on bone tissue. In fact, in culture, Yamaguchi and Gao[175] reported that the antiestrogen tamoxifen blocks the ability of genistein to inhibit parathyroid-induced bone resorption. However, they do indicate that isoflavones are tissue selective. Many researchers in this area, thus, consider soy isoflavones as naturally occurring selective estrogen receptor modulators (SERMs).

Several human studies found that bone resorption markers were decreased relative controls;[101–105] however, other findings suggest soy may also stimulate bone formation.[104,105] Interestingly, in rodents, Ishida and colleagues[85] found that while both daidzin and genistin retarded bone loss, daidzin but not genistin inhibited bone turnover induced by ovariectomy. Thus, genistin appeared to retard bone loss by increasing bone formation. Fanti and colleagues[84] suggested that genistein stimulates bone formation by suppressing the activity of one or more cytokines, whereas Blair and colleagues[82] suggested that genistein may suppress osteoclastic activity through its ability to inhibit tyrosine kinases.

Potentially important insight into the action of genistein comes from a study that found that, in culture, blocking the action of transforming growth factor-β (TGFβ) has been shown to prevent the inhibitory effects of estrogen on bone breakdown.[76] Kim and colleagues[75] have found that, at least in breast cells, genistein increases TGFβ levels.

Finally, as noted at the outset, the soybean isoflavones have a similar chemical structure to the synthetic isoflavone ipriflavone, which has been shown to be quite effective in inhibiting bone loss in perimenopausal and postmenopausal women.[35–37] In fact, daidzein is a metabolite of ipriflavone. There are data indicating that ipriflavone favorably affects both bone resorption and bone formation.[180,181] However, the standard dose of ipriflavone is 600 mg/day and daidzein does not appear to be one of the metabolites of ipriflavone responsible for its effects on bone.[182,183] Interestingly, Ishida and colleagues[85] found that while both genistin and daidzin retarded bone loss in ovariectomized rats, ipriflavone was without effect. Thus, the extent to which insight on the mechanism

of action of isoflavones on bone tissue can be gained by looking at ipriflavone is not clear. Clearly though, there are data indicating that isoflavones may both inhibit bone resorption and stimulate bone formation and that the proposed mechanisms for the effects of isoflavones on bone tissue include both estrogenic and nonestrogenic effects.

XIV. CONCLUSIONS AND PUBLIC HEALTH IMPLICATIONS

Overall, the evidence that soy protein and/or its isoflavones favorably affect BMD is promising. However, because of the limited data, no firm conclusions can be made at this time. Fortunately, several human studies are under way. Therefore, considerably more knowledge about this area of research will be available within just a few years.

Thus far, with only one exception, the existing human studies are supportive of protective effects of soy with studies having only been conducted in women. Nevertheless, the strength of the evidence clearly justifies conducting long-term human studies using either isolated isoflavones or soyfoods. Arguments can be made in favor of each approach. Isoflavone supplements may allow for better compliance and for less confounding by other dietary variables as when soyfoods are added to the diet. Data clearly suggest isoflavones are the primary components of soy acting on bone tissue. However, to date, human studies have used only soy protein. Moreover, the use of soy protein allows for the possibility that nonisoflavone components of soy, although unlikely, contribute to the bone-related effects. Therefore, before conducting long-term human trials using isolated isoflavones, the effects of using isoflavone supplements vs. soy protein on bone turnover and BMD should be directly compared in short-term studies.

Should longer-term studies demonstrate that soy and/or its isoflavones favorably affect BMD, studies comparing fracture incidence will be needed. The results of these studies will likely not be known for at least 5 to 10 years. However, because of the many hypothesized health benefits of soyfoods and their favorable nutrient profile, the public should still be encouraged to incorporate soyfoods into their diet. Those individuals replacing dairy milk with soy milk should be advised to consume calcium-fortified products. Calcium bioavailability from soyfoods is equal to that from dairy milk.[184]

Soyfoods cannot be recommended at the present time as a substitute for estrogen replacement. But soyfoods can be strongly recommended for those women who choose not to use estrogen. How much soy should be consumed? Few dose–response studies have been conducted. Nevertheless, human data suggest 60 to 90 mg of isoflavones per day may be needed, or about two to three servings of traditional soyfoods. From a practical perspective, incorporating an amount of soy into the diet that will provide this level of isoflavones will be a challenge for many consumers. However, the food industry is responding with new soy products that should make such dietary changes possible. Furthermore, it may be that lesser amounts of soy protein or soy isoflavones consumed over the course of a lifetime will exert favorable effects on bone tissue. This remains to be determined.

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6 Phytoestrogens: Involvement in Breast and Prostate Cancer

Julie H. Mitchell

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I. INTRODUCTION

The increased awareness of the role that diet plays in human and animal health has led to the recognition that some non-nutrient components of foods may provide protection against many Western diseases, including hormone-dependent cancers, colon cancer, and coronary heart disease. Phytoestrogens are ubiquitous in the plant kingdom with isoflavones and lignans being the main classes of current interest in human nutrition. These compounds, which occur mainly in soybean and whole-grain products, are similar in structure and molecular weight to endogenous steroidal estrogens and thus have the ability to bind to estrogen receptors and elicit hormonal responses in certain mammalian tissues. Furthermore, non-receptor–related functions for phytoestrogens include effects on sex hormone metabolism and bioavailability, protein synthesis, and growth factor action (Table 6.1). Moreover, their ability to moderate malignant cell proliferation makes them strong candidates as chemoprotective agents. This chapter discusses the biological activities of phytoestrogens with reference to breast and prostate cancers and reviews the evidence linking them to phytoestrogen intake.

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TABLE 6.1
Anticancer Effects of Phytoestrogens

Anticancer mechanisms of phytoestrogens	Ref.
Inhibition of protein tyrosine kinases	21
Inhibition of aromatase enzyme	17, 18
Inhibition of 17β hydroxysteroid dehydrogenase	19, 20
Inhibition of 5α-reductase	20
Increased synthesis of sex hormone binding globulin	9, 10
Increased menstrual cycle length	14, 59
Inhibition of tumour cell invasion	77
Inhibition of angiogenesis	93
Antioxidant effects	29, 94

II. MECHANISMS OF ACTION OF PHYTOESTROGENS

A. Estrogen Receptors

Phytoestrogens bind to estrogen receptors (ERs) and via estrogen response elements may stimulate the transcription of cell-specific genes. However, by competing with estradiol (E_2) for binding sites, they may also act as estrogen antagonists reducing the availability of E_2 in target cells. These estrogen agonist/antagonist effects depend on a number of factors including phytoestrogen concentration, number of ERs, and the level of endogenous estrogens. Cell culture studies indicate that phytoestrogens have a biphasic effect on growth of human breast cancer cells *in vitro*. Physiological concentrations (1 n*M* to 10 μ*M*) of genistein, daidzein, equol, coumestrol, and the lignan enterolactone stimulate the growth of MCF-7 (ER+) cells in the absence of endogenous estrogens.[1–4] The effects of phytoestrogens on cell growth in the presence of E_2 are variable and depend on concentration and relative binding affinity to the ER.[2,3,5] Regardless of the presence of E_2 or growth factors, high concentrations of phytoestrogens (>10 μ*M*) cause growth inhibition in both ER+ and ER– cell lines suggesting a nonreceptor-related mechanism.[6] The discovery of a second estrogen receptor ERβ, with a different tissue distribution from that of the classical ERα (Figure 6.1), further complicates our understanding of phytoestrogen actions.[7] In addition, many phytoestrogens have a stronger binding affinity for ERβ than ERα which may help explain the different responses of compounds in various tissues.[8]

B. Sex Hormone Metabolism

Cancers of the breast and prostate are initially hormone dependent, so any compounds that can alter either the metabolism or bioavailability of sex hormones may influence the development of these diseases. Phytoestrogens stimulate the synthesis of sex hormone binding globulin (SHBG) *in vitro*, and in epidemiological studies urinary excretion of phytoestrogens correlates positively with plasma SHBG concentrations and negatively with percentage free E_2 and testosterone concentrations.[9,10] In addition, higher levels of SHBG and decreased levels of free E_2 and testosterone occur in vegetarians who have a lower incidence of breast and prostate cancers than non-vegetarians.[11] Despite this, a number of short-term phytoestrogen supplementation studies found no change in plasma SHBG concentrations.[12–15]

The enzyme aromatase catalyzes the conversion of androgens to estrogens, and aromatase inhibitors are commonly used in the treatment of breast cancer, particularly for postmenopausal women where aromatization of androgens is a major source of estrogen.[16] The lignan enterolactone as well as some flavonoid compounds are moderate or weak inhibitors of aromatase *in vitro*, suggesting a possible role in chemoprevention.[17,18] A number of phytoestrogens inhibit

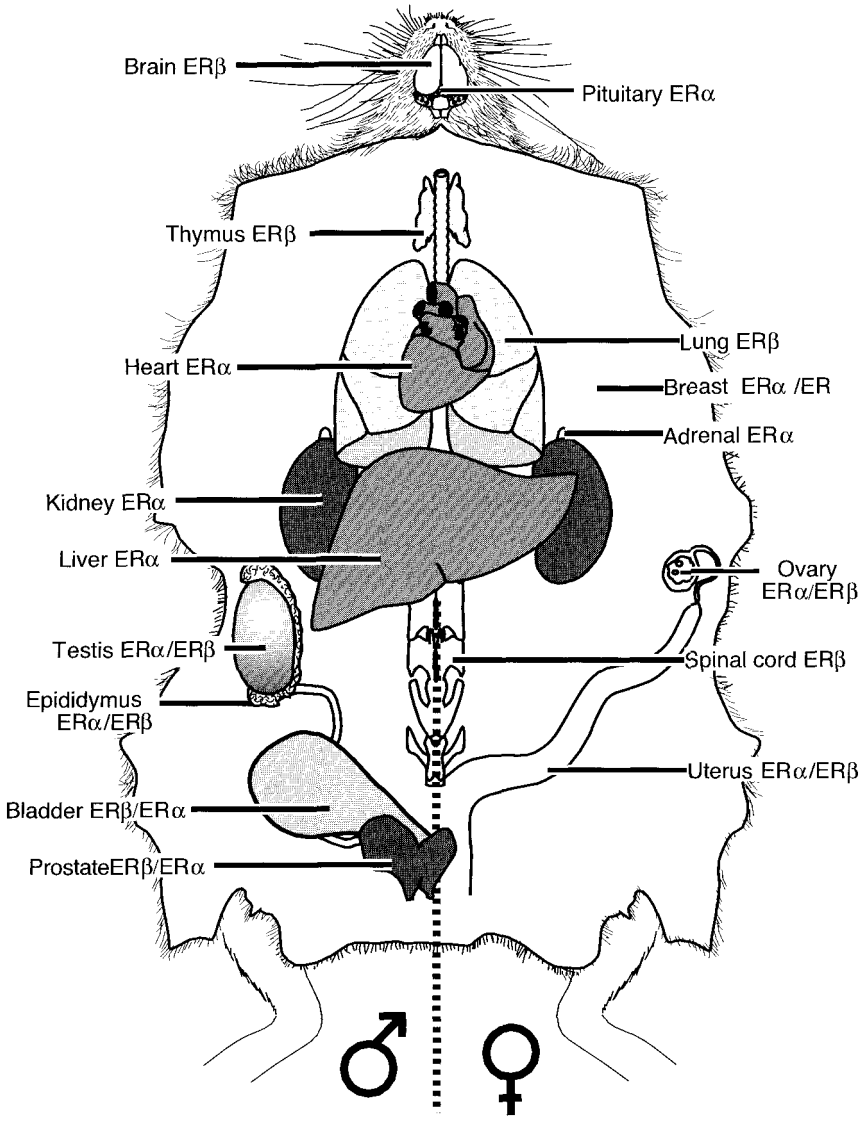

FIGURE 6.1 Schematic diagram illustrating the tissue distribution of ERα and ERβ in the male and female rat.

17β-hydroxysteroid dehydrogenase and 5α-reductase enzymes, which catalyze the conversion of estrone to the biologically active E_2 and testosterone to the biologically active 5α-dihydrotestosterone (DHT), respectively.[19,20]

C. Protein Tyrosine Kinases, Growth Factors, and Other Enzymes

Protein tyrosine kinases (PTKs) play an important role in cell proliferation by the tyrosine autophosphorylation of many growth factor receptors, including those for epidermal growth factor (EGF), insulin, insulin-like growth factor, and platelet-derived growth factor. In addition, PTKs are associated with oncogene products of several retroviral families involved in cell transformation.[21] The isoflavone genistein was originally thought to inhibit the autophosphorylation of the EGF receptor *in vitro*, providing a mechanism for the inhibition of cell proliferation.[21] However,

subsequent studies using prostate and breast cancer cells *in vitro* concluded that growth inhibition by genistein did not depend on inhibition of EGF receptor activation but on other pathways such as influences in signal transduction.[22,23] In support of this, genistein supplementation has been shown to downregulate EGF receptor expression in rat dorsolateral prostate.[24]

DNA topoisomerases are involved in DNA replication and repair and thus play a key role in cell proliferation and differentiation. They function in DNA cleavage and religation forming an intermediate "cleavable complex" between DNA and enzyme. Topoisomerase inhibitors can be used as cancer therapeutic agents, acting by the stabilization of the "cleavable complex." The isoflavone genistein, as well as some other flavonoid compounds, can inhibit topoisomerases I and/or II *in vitro* providing a further anticancer mechanism.[25] Genistein induces dose-dependent strand breakage to DNA in prostate tumor cells in culture.[26]

Antioxidants may protect against cancer by removing reactive oxygen species before they have a chance to induce cellular damage.[27] Although many plant polyphenols have marked antioxidant capacity, and isoflavones have limited antioxidant ability *in vitro* and in human volunteers, a soy supplement did not alter the antioxidant capacity of plasma.[28,29]

A novel anticancer role of genistein in cell culture systems involves inhibition of cell growth and induction of apoptosis by modulating transforming growth factor-β (TGF-β) signaling pathways.[30,31]

III. BREAST CANCER

A. Introduction

Breast cancer remains the most common cancer in women in Northern Europe and the United States. Established risk factors mainly relate to reproductive events. For example, risk is increased by early menarche, late onset of natural menopause, nulliparity, late age at first birth, and hormone replacement therapy.[32,33] Rapid growth rate early in life, determined in part by nutritional factors, leads to earlier onset of puberty and thus increased risk.[34,35] Breast-feeding appears to reduce the risk of breast cancer as a large proportion of epithelial cells in the breast, the targets for carcinogens, undergo differentiation to milk-producing cells.[36] The major link between all the above risk factors is estrogen exposure over a lifetime, so it was initially thought that environmental estrogens would increase the risk of breast cancer. However, investigations into the estrogen-antagonist effects of exogenous compounds as well as the recent discovery of the ERβ, with different binding affinities and tissue distribution from ERα, have helped clarify the roles of compounds such as phytoestrogens in different tissues.[7,8,37]

B. *In Vitro* Studies

Phytoestrogens have a biphasic effect on ER+ breast cancer cell growth *in vitro*. At concentrations similar to those reported in human plasma (< 10 μ*M*), isoflavones and lignans act as weak estrogens and can induce expression of the estrogen-regulated antigen pS2 as well as stimulating cell growth in the absence of E_2.[2,3,38,39] In the presence of low concentrations of E_2 (0.01 n*M*) isoflavones (≤10 μ*M*) further stimulate the E_2-induced growth of breast cancer cells;[4,40] however, with higher concentrations of E_2, phytoestrogens inhibit E_2-induced cell growth.[2,23] Thus, it seems that in the presence of low concentrations of E_2, the ERs are not fully saturated allowing phytoestrogens to have an additive effect. Higher E_2 concentrations, which may fully saturate ERs, cause phytoestrogens to compete with E_2 for ER binding and thus reduce the E_2 effects on cell growth (Figure 6.2). In support of this, 10 μ*M* genistein increased DNA synthesis induced by 0.01 n*M* E_2 in MCF-7 cells but had no effect on DNA synthesis induced by 0.1 n*M* E_2 and inhibited DNA synthesis induced by 1 n*M* E_2.[4] Interestingly, the concentrations of estrogens in breast ductal fluid is up to 40 times higher than in serum,[41] increasing the likelihood of phytoestrogen effects being protective. Long-term exposure of breast cancer cells to isoflavones reduced ER mRNA levels in MCF-7 cells, further emphasizing that phytoestrogens may protect against estrogen action.[3]

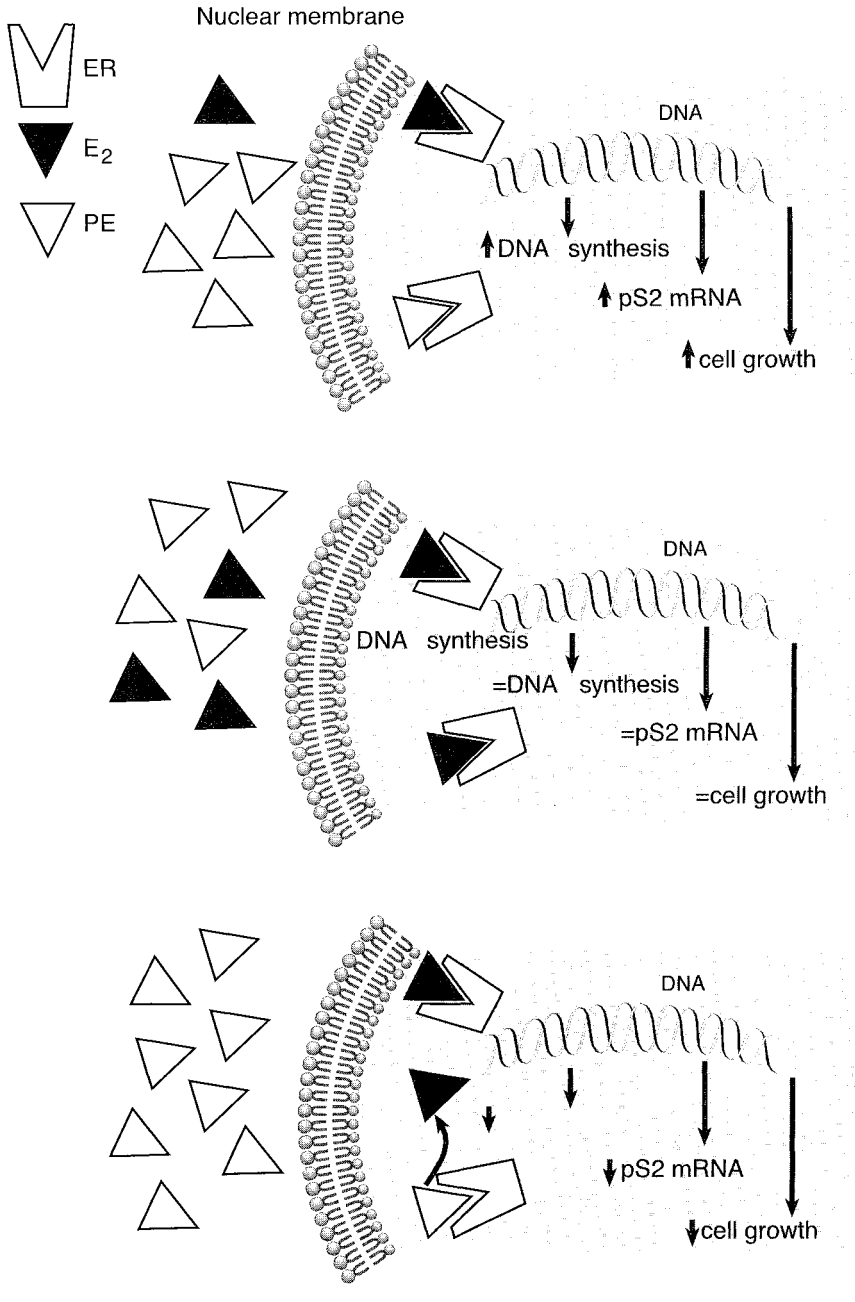

FIGURE 6.2 Mechanism by which phytoestrogens may interact with endogenous estrogen to activate ERs and affect cell growth in breast cancer cells. Upper panel: In the presence of low estradiol (E_2) concentrations, phytoestrogens (PE) may bind to available ERs and stimulate cell growth. Middle panel: Where phytoestrogens and E_2 are present in similar concentrations, phytoestrogens would be unable to compete successfully with E_2 for receptor binding and may not affect E_2-stimulated cell growth. Bottom panel: Phytoestrogens in great excess to endogenous estrogen may displace E_2 from ERs.

High concentrations of phytoestrogens inhibit cell growth in ER+ and ER– cell lines and inhibit the E_2-induced expression of pS2 by non-ER–related mechanisms or by downregulation of ER mRNA expression.[23,40,42,43]

C. Animal Studies

The effects of phytoestrogen intake, by dietary means or subcutaneous injection, on mammary carcinogenesis have been investigated in numerous animal studies with conflicting results depending on dose and timing of exposure. Subcutaneous injection of genistein into neonatal and prepubertal rats protects against chemically induced mammary carcinogenesis in adulthood.[44,45] This protective effect was due to altered gland morphology, genistein treatment enhancing gland differentiation, and promoting maturation of undifferentiated terminal end ducts, which are more susceptible to chemical carcinogens, to the more differentiated lobules.[46] However, the levels of genistein were extremely high (approximately 5 mg in neonatal rats and 15 mg in prepubertal rats) and caused adverse effects on the reproductive/endocrine system in neonatal treated animals.[47] A number of studies attempting to show that soy-based diets or phytoestrogens as pure compounds have protective effects against chemically induced mammary carcinogenesis in rodents invariably did not achieve statistical significance.[48–53] Lignan precursors from flaxseed protected against chemically induced mammary carcinoma when the lignan was given for a 20-week period after the chemical treatment.[54] The lignan dose was nontoxic in adult rats when begun after weaning, but high lignan consumption preweaning, i.e., exposure during pregnancy and lactation, caused increases in sex organ weights, and increased testosterone and E_2 levels in male and female offspring.[55,56] One recent study has reported that genistein increases mammary cancer risk.[57] Pregnant dams exposed, by subcutaneous injection to levels of genistein which approximate human intake, produced offspring which were more susceptible to DMBA-induced tumors. However, a suboptimal dose of DMBA was used and tumor incidence varied between 20 and 50% in control animals, thus confounding interpretation of results.

D. Human Intervention Trials and Epidemiology

Well-controlled studies investigating the hormonal effects of phytoestrogens in women are uncommon, since high isoflavone/lignan diets can be unpalatable, making compliance difficult, and investigations are often invasive and of a sensitive nature. In addition, those trials that have been conducted give conflicting results possibly because of differences in duration and phytoestrogen dose. Soy supplementation increased menstrual cycle length in two studies by increasing follicular phase length.[14,58] Breast cells proliferate more rapidly during the luteal phase and so an increased follicular phase length will reduce the number of luteal phases (progesterone exposure) during a lifetime and could decrease breast cancer risk. In one study, the mid-cycle peaks of both follicle stimulating hormone (FSH) and luteinizing hormone (LH) were suppressed; however, E_2 concentrations were higher during the follicular phase.[14] Longer-term soy supplementation modified estrogen metabolism to more biologically preferable end points and a decreased estrogen synthesis was suggested due to inhibition of hormone-metabolizing enzymes such as aromatase and 17β-hydroxysteroid dehydrogenase.[59] In contrast, flaxseed lignan supplementation had no effect on menstrual cycle length and increased luteal phase length in women.[13] Furthermore, two recent studies have reported that soy supplementation in premenopausal women has a stimulatory effect on breast tissue as assessed by elevated serum E_2 levels, increased nipple aspirate fluid (NAF) volume, an elevated number of proliferating epithelial cells and appearance of hyperplastic epithelial cells, and an increased number of progesterone receptors.[60,61] NAF volume and hyperplastic epithelial cell numbers are correlated with breast cancer risk;[62] thus, further longer-term studies are required to investigate these effects fully. In postmenopausal women NAF volume and serum hormone levels were unaffected, but a small estrogenic effect on vaginal cytology was observed.[15,60,63]

A number of epidemiological studies have associated soybean intake with reduced breast cancer risk in Asian populations. In a prospective study over 17 years, a significant graded correlation was noted between soybean paste soup consumption and breast cancer risk in Japan.[64] Similarly, case-control studies in Singapore and Japan associated regular intake of soybean foods

with decreased breast cancer risk in premenopausal but not postmenopausal women.[65,66] In contrast, no association between soy intake and breast cancer risk was detected in a large case-control study in two areas of China.[67] In Asian–Americans, tofu intake was protective against breast cancer in both pre- and postmenopausal women; however, this was the case only for Asians who migrated to United States and not Asians born in the United States.[68,69] Thus, the evidence supporting a link between phytoestrogen intake and breast cancer risk is substantial for Eastern populations but is limited for Western societies with a high rate of cancers. Nevertheless, a case-control study in Australia showed an inverse correlation between urinary excretion of isoflavones and lignans and breast cancer risk.

E. Summary

Epidemiological evidence relating phytoestrogen intake with reduced breast cancer risk in Asian women is robust. Yet, there is little evidence to support the theory that increasing phytoestrogen intake in Western women will be protective; indeed, such intakes may even cause adverse effects. Administration of high doses of phytoestrogens by subcutaneous injection inhibits chemically induced mammary carcinogenesis in rats, but causes adverse effects on the reproductive system when administered in the neonatal period.[46] Furthermore, dietary administration of high phytoestrogen doses to young animals caused changes in sex organ weights and hormone levels[57] which may ultimately make them more susceptible to cancer. The increase in menstrual cycle length due to soy feeding may be anticarcinogenic since the fewer cycles over a lifetime would reduce the overall exposure to estrogens. However, this may be counteracted by higher E_2 concentrations during the lengthened follicular phase. The longer menstrual cycle length reported for Eastern women may be a protective factor against breast cancer,[71] but they also have 20 to 30% lower serum E_2 concentrations than Western women.[72] Of particular concern are the human intervention trials whereby soy feeding increased serum E_2 concentrations and had a stimulatory effect on premenopausal breast tissue. The consequence of this apparent estrogenic action requires further investigation.

IV. PROSTATE CANCER

A. Introduction

Prostate cancer is one of the most common cancers to affect males in Western countries, while in Africa, Eastern Europe, and Japan the risk of this disease remains low.[73,74] However, the prevalence of latent cancers, discovered at post-mortem, is similar between high- and low-risk populations with genetic and lifestyle factors being implicated in the progression to the malignant form of the disease.[75] The marked increase in prostate (and breast) cancer incidence in migrant populations relocating from low- to high-risk geographic areas[76] suggests that environmental factors such as diet play a major role in hormone-dependent cancer etiology. Thus, high soybean intake may be one factor contributing to the low prostate cancer mortality in Eastern populations although evidence to date is equivocal.

B. *In Vitro* Studies

Peterson and Barnes[22] showed that genistein and its metabolic precursor biochanin A inhibit the growth of human prostate tumor cell lines in culture. This indicated that soy may be an important nutritional factor in accounting for the low rate of prostate cancer in Asian men. The mechanism of action may involve topoisomerase inhibition since genistein added to culture medium induced DNA strand breakage in androgen receptor (AR)-positive and AR-negative human prostate tumor cell lines.[26] Genistein also inhibited the invasion potential of human prostate cancer cells in culture by inhibiting the expression of tumoral proteinases.[77] The concentrations of isoflavones used in

these experiments were higher than those reported for plasma in the population of soy-consuming nations. However, levels of phytoestrogens may be higher in prostatic fluid than in plasma and there are no reports of the maximum concentrations that occur in cells.[78,79]

Isoflavones and lignans (individually and as a mixed cocktail) inhibited the activities of 5α-reductase enzymes in genital skin fibroblasts and prostate tissue homogenates providing a possible mechanism for the chemoprotective effect of soy against prostate cancer.[20] In support of this, lower levels of markers of 5α-reductase activity are reported in Japanese males who also have a lower incidence of prostate cancer than U.S. males.[80] Further evidence for a role of phytoestrogens in prostate cancer etiology was recently provided when genistein was shown to inhibit the growth of benign prostate hypertrophy (BHP) and prostate cancer tissue in histoculture.[81]

C. Animal Studies

The number of animal studies associating phytoestrogens with prostate cancer are limited and the fact that many are abstracts makes review more difficult. Genistein-supplemented diets reduce the growth and development of human prostate tumor cells (LNCaP) implanted into nude/immunodeficient mice.[82,83] Subcutaneous injections of genistein suppressed the growth of tumors from tumor cell implants in rats[85] and a soybean flour diet retarded the growth of implanted prostate tumors in rats as compared with animals fed a control diet. However, the concentrations of genistein used in most of these experiments were high (Table 6.2)[84] and the data are only available in abstract form. In contrast, low levels of genistein (which more closely relates to human dietary consumption) in drinking water had no inhibitory effect on the growth of MAT-Lylu rat prostate cancer cells implanted subcutaneously.[86] A similar genistein dose administered by subcutaneous injection caused slight but nonsignificant

TABLE 6.2
Estimation of Equivalent Dietary Intakes of Phytoestrogens per Body Weight in Humans and Rodents

- The total phytoestrogen consumption in Eastern populations or in adults taking dietary phytoestrogen supplements may be approximately **60 to 75 mg per day**. Therefore an average 60- to 75-kg adult would consume approximately 1 mg phytoestrogen per kg body weight (bw).
- An adult rat weighs approximately 300 g and consumes approximately 30 g diet per day. To consume 1 mg phytoestrogen per kg bw equivalent to human Eastern population the rat diet would contain approximately 10 mg phytoestrogen per kg diet to allow a total intake of **0.3 mg per day.**
- An adult mouse weighs approximately 35 g and consumes approximately 3.5 g diet per day. To consume 1 mg phytoestrogen per kg bw equivalent to human Eastern population the mouse diet would contain approximately 10 mg phytoestrogen per kg diet to allow a total intake of **0.035 mg per day.**

Ref.	Animal	Phytoestrogen Exposure	Method	Approx. Total Intake (mg)
86	Rat	0.285 mg/kg bw	Drinking water	0.1
86	Rat	0.428 mg/kg bw	Subcutaneous injection	0.2
85	Rat	50 mg/kg bw	Subcutaneous injection	15
84	Rat	330 mg/kg diet[a]	Dietary intake	1
87	Rat		Dietary intake	3.8
24	Rat	1 g/kg diet	Dietary intake	30
82	Mouse	1 g/kg diet	Dietary intake	3.5

[a] Diets contain 330 g soy flour per kg diet. Calculations based on an estimated isoflavone of soy flour of 1 mg/g.

reduction in tumor weight. Dietary isoflavones (genistein and daidzein) reduced the incidence and increased the latency period of chemically induced prostate tumors in rats when the supplement was started before the methynitrosuria (MNU) induction.[87] When the isoflavone supplement was provided after the MNU treatment the results were less significant. The authors suggest that the MNU treatment was too intense for the modest dietary supplement to be effective and that the increased latency period will have more significance in the development of spontaneous tumors. The mechanism of action may relate to impaired cell signaling since genistein can downregulate the expression of EGF receptor and other phosphorylated proteins in the rat dorsolateral prostate.[24] This is the first study to report genistein inhibition of a signal transduction pathway *in vivo*.

D. Human Intervention Trials and Epidemiology

A modern database used to analyze dietary intakes of individual phytoestrogens in patients with prostate cancer and controls indicated a significant protective effect of genistein, daidzein, and coumestrol.[88] Furthermore, a 66-year-old prostate cancer patient in Australia took a 160-mg phytoestrogen supplement daily for 1 week prior to radical prostatectomy. On histological examination of the prostatectomy specimen, compared with the preoperative needle biopsy, significant apoptosis in tumor cells suggestive of tumor regression was evident.[89] In support of this, higher concentrations of the isoflavones daidzein and equol were found in prostatic fluid of men from Hong Kong than in men from Britain where the risk of prostate cancer is higher.[78]

In contrast, the epidemiological evidence associating phytoestrogen intake with prostate cancer risk is largely negative, albeit based on only a few studies. In two studies involving 100 to 200 volunteers there was no significant association between soybean paste/miso soup consumption and prostate cancer risk.[90,91] A further large-scale study using 7999 men (of Japanese ancestry) in Hawaii also found no correlation between miso soup intake and prostate cancer risk but a reduced risk of prostate cancer risk with tofu intake; however, the correlation was of marginal significance ($P = 0.054$).[92]

E. Summary

Many *in vitro* studies emphasize the ability of phytoestrogens to inhibit prostate cancer cell growth and provide possible chemoprotective mechanisms. Yet, the concentrations of compounds used to achieve such results are invariably higher than can be achieved *in vivo*. A similar comment could be made about many of the animal studies, although a direct comparison of intake between species (Table 6.2) is very crude. Naik and colleagues[86] published the only work reviewed in this chapter to use phytoestrogen supplementation to a level equivalent to human consumption and this was the only study to show no protection against tumor growth. Genistein administered by subcutaneous injection showed a minor but nonsignificant inhibition of tumor growth over a very short period. That dietary isoflavones reduced the incidence and increased the latency period of induced tumors in rats if the animals were supplemented before tumor induction is indicative of a protective effect.[87] However, perhaps a more relevant experiment would be to determine if physiological concentrations of phytoestrogens in the diet of Lobund-Wistar rats could reduce the risk of spontaneous prostate tumors. The epidemiological studies show little association between phytoestrogen intake and prostate cancer risk, although estimation of phytoestrogen concentration in foods and biological fluids was of limited accuracy. The most recent study, using modern analytical technology, found a significant correlation between isoflavone intake and prostate cancer, and the Australian case-study showing tumor cell apoptosis after phytoestrogen supplementation certainly invites further investigation.[88,89]

V. DISCUSSION

Research into the actions of phytoestrogens has demonstrated a number of chemopreventive properties; yet, their actions *in vivo* appear to be largely estrogenic. Genistein has only about 1/1000th

the estrogenic activity of E_2; yet its circulating concentration in individuals consuming a moderate amount of soy foods may be 1000-fold higher than peak levels of endogenous estrogens. Furthermore, there is little information regarding whether different phytoestrogen compounds might have an additive effect or alternatively compete for receptor binding sites.

Evidence from animal studies and human intervention trials supports a role for phytoestrogens in prostate cancer incidence, but epidemiological studies are less positive. Data associating breast cancer incidence with phytoestrogen intake are conflicting, with both animal and human intervention trials documenting adverse effects on hormone levels and breast tissue. Furthermore, the protective effect of soy intake against breast cancer was only evident in Asian immigrants in United States and not in American-born Asians.[70] The types of soybean foods eaten in Asian countries are different from the more processed foods in the United States and Britain, and differences in gut microflora between such different populations will affect the metabolism and distribution of both isoflavones and lignans. Nevertheless, Eastern populations consume several times the amount of phytoestrogen-containing foods than populations in Western countries and have lower incidences of breast and prostate cancer.

ACKNOWLEDGMENTS

This work was supported by the Ministry of Agriculture, Fisheries, and Food (MAFF)/Food Standards Agency (FSA) and the Scottish Office Agriculture Environment and Fisheries Department (SOAEFD).

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7 Anticancer and Cholesterol-Lowering Activities of Citrus Flavonoids

Najla Guthrie and Elzbieta M. Kurowska

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I. INTRODUCTION

Flavonoids are a group of polyphenolic compounds ubiquitous in many plants including fruits, vegetables, nuts, seeds, grains, tea, and wine.[1] Over 6000 different flavonoids have been described.[2] They occur as the free forms, glycosides, and methylated derivatives. Citrus flavonoids are a major class of secondary metabolites that have important biological activity.[3] These secondary metabolites are found in citrus fruit at relatively high levels and their chemistry and properties are well described.[4] Flavonoids from citrus are benzo-γ-pyrone derivatives and belong largely to two classes: flavanones and flavones (Figure 7.1). The most prevalent flavanones are hesperetin from oranges and naringenin from grapefruit, both found in the fruit tissue and peel largely as their glycosides, hesperidin and naringin. Hesperidin is responsible for the cloudy appearance of orange juice due to its poor solubility in water and naringin is one of the main bitter principles in grapefruit. Relatively common in citrus are also two polymethoxylated flavones, tangeretin and nobiletin, both present in tangerines.[4]

Dietary intake of citrus flavonoids is substantial, especially in countries with high consumption of citrus juices. However, the bioavailability of these compounds is still poorly understood. Recent studies demonstrated that in humans, the free forms of hesperidin and naringin present in citrus juices can be absorbed into the blood system[5] most likely following their liberation from the glycosides by intestinal bacteria.[6] Some flavonoids, especially those possessing methoxy groups, such as hesperetin and polymethoxylated flavonoids, were also postulated to remain longer in the body due to their facilitated uptake by cells.[7]

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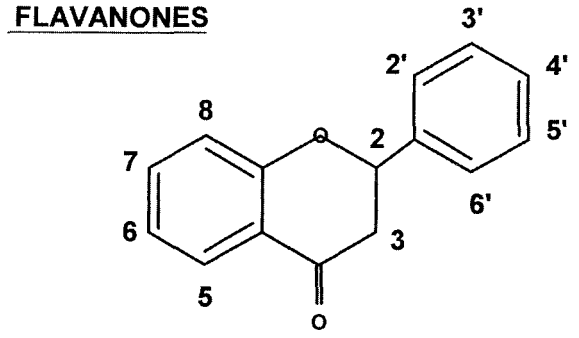
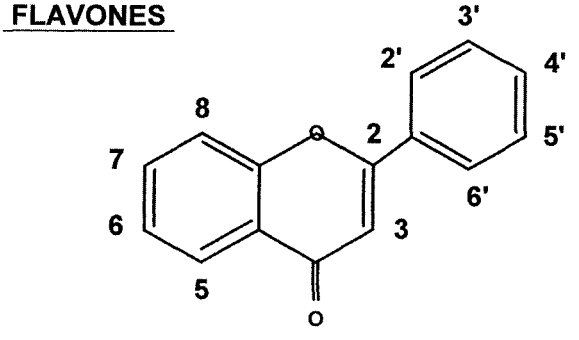


	5	7	3'	4'
HESPERETIN	OH	OH	OH	OCH_3
NARINGENIN	OH	OH	—	OH



	5	6	7	8	4'	5'
TANGERETIN	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3	—
NOBILETIN	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3

FIGURE 7.1 Formulae of flavonoids.

The role of dietary citrus flavonoids in human health is the object of growing scientific interest. The beneficial effects reported over the past 7 years include antiallergic, anti-inflammatory, antihypertensive, and diuretic, as well as anticancer and hypolipidemic properties.[8–15] This chapter focuses on the authors' recent *in vitro* and *in vivo* experiments aimed to investigate the anticancer and cholesterol-lowering potential of citrus juices and the principal citrus flavonoids.

II. CITRUS FLAVONOIDS AND CANCER

Breast cancer is the most prevalent cancer in women of developed countries, and its incidence has been increasing worldwide.[16] Attempts to improve survival and reduce the risk of relapse following diagnosis have shown limited success; thus, there is still substantial room for improvement.

Although researchers are evaluating new drugs, another promising approach is the investigation of dietary components as anticancer agents.

Epidemiological studies on diet and cancer have provided leads in the search for naturally occurring anticancer agents.[17] There is general agreement that plant-based diets, rich in whole grains, legumes, fruits, and vegetables, reduce the risk of various types of cancer, including breast cancer.[18] A variety of compounds produced by plants have been investigated for their anticancer activity.[19] These include the flavonoids, which are an integral part of the human diet. Our interest in the anticancer properties of citrus flavonoids began with the observation that naringenin inhibited proliferation and growth of MDA-MB-435 estrogen receptor-negative (ER–) human breast cancer cells in culture more effectively than did genistein.[20] This led us to conduct further studies, which have produced a number of important observations.

A. Cell Culture Studies

1. Effects on Estrogen Receptor-Negative Cells

Citrus flavonoids were investigated for their effects on proliferation of MDA-MB-435 ER– human breast cancer cells in culture.[21] The IC_{50} (concentration that inhibits cell proliferation by 50%) values are presented in Figure 7.2. Hesperetin, the aglycone of the flavonoid present in oranges, was found to inhibit ER- human breast cancer cells as effectively as did naringenin (Figure 7.2). Two other citrus flavonoids, tangeretin and nobiletin, found in tangerines, were much more effective at inhibiting the proliferation of these cells (Figure 7.2).

The ability of citrus flavonoids to inhibit cell growth was also investigated by treating the cells at their IC_{50} concentrations and following the growth of the cells over a 10-day period.[21] They inhibited the growth of these cells and the effect was apparent after 2 days of treatment.[21] Cytotoxic

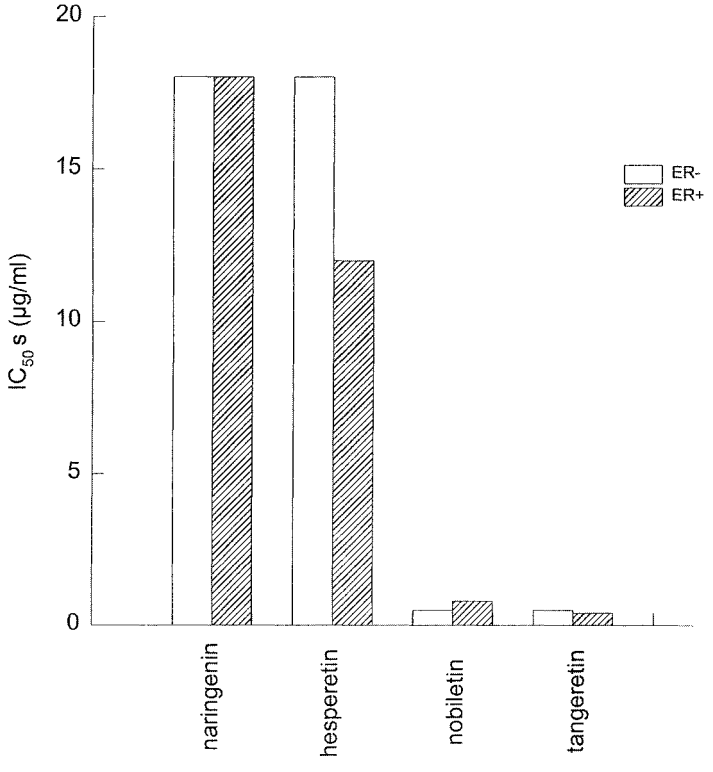


FIGURE 7.2 Inhibition of ER– and ER+ human breast cancer cells by citrus flavonoids.

effects of the citrus flavonoids were investigated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolum bromide (MTT) assay.[22] Most of the cells were viable at IC_{50} concentrations[21] indicating that the antiproliferative activity of the compounds was not due to nonspecific cytotoxicity.

2. Effects on Estrogen Receptor-Positive Cells

The effects of citrus flavonoids on the proliferation, growth, and viability of MCF-7 ER+ human breast cancer cells were also investigated.[23,24] The IC_{50} values are presented in Figure 7.2. Tangeretin and nobiletin were again the most effective inhibitors, with IC_{50} values of 0.8 and 0.4 μg/ml, respectively (Figure 7.2). Further studies were performed to investigate whether this inhibition was due to the flavonoids acting as antiestrogens. MCF-7 cells were depleted of all endogenous steroids and treated with flavonoids or tamoxifen (a drug widely used in the treatment of hormone-responsive breast cancer) in the absence or presence of 100 n*M* estradiol as previously described[24]). The results (Figure 7.3) show that the inhibition by all the citrus flavonoids was unaffected by estradiol in contrast to the results with tamoxifen.

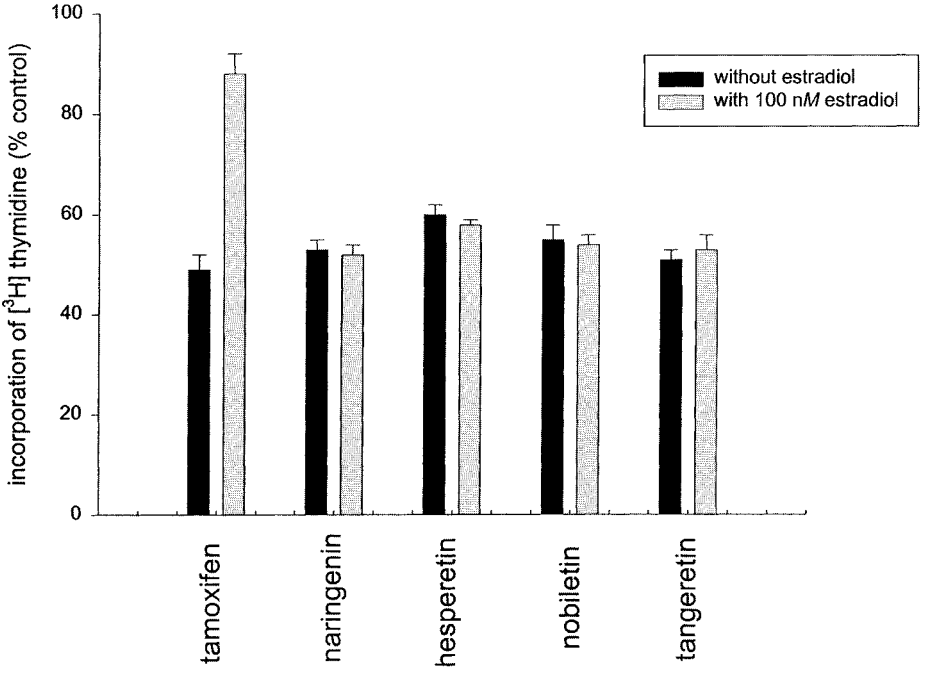


FIGURE 7.3 Inhibition of proliferation of MCF-7 human breast cancer cells by tamoxifen and by citrus flavonoids in the presence and absence of excess estrogen.

3. Synergistic Effects

In other studies with ER– and ER+ human breast cancer cells *in vitro*, we have observed that 1:1 combinations of citrus flavonoids with tocotrienols (a form of vitamin E) (Tables 7.1 and 7.2) or tamoxifen and 1:1 combinations of tocotrienols with tamoxifen (Tables 7.3 and 7.4) inhibit proliferation of the cells more effectively than the individual compounds by themselves. The most effective combination with ER– cells was tangeretin and γ-tocotrienol (IC_{50} 0.05 μg/ml)[25] (Table 7.1). With ER+ cells, the best results were obtained with tangeretin and γ-tocotrienol (IC_{50} 0.02 μg/ml) (Table 7.2), nobiletin + tamoxifen (IC_{50} 0.4 μg/ml) (Table 7.4), and δ-tocotrienol + tamoxifen (IC_{50} 0.003 μg/ml) (Table 7.4).[25] When combined in 1:1:1 combinations of flavonoids, tocotrienols,

TABLE 7.1
Synergistic Effects of Flavonoids and Tocotrienols on Inhibition of Proliferation of MDA-MB-435 ER– Human Breast Cancer Cells in Culture [IC_{50} (μg/ml)]

	Tocotrienols			
Flavonoid	**None**	**α**	**δ**	**γ**
None		90	90	30
Naringenin	18	8	1	4
Hesperetin	18	2	19	19
Tangeretin	0.5	0.1	0.1	0.05
Nobiletin	0.5	2	0.25	0.5

TABLE 7.2
Synergistic Effects of Flavonoids and Tocotrienols on Inhibition of Proliferation of MCF-7 ER+ Human Breast Cancer Cells in Culture [IC_{50} (μg/ml)]

	Tocotrienols			
Flavonoid	**None**	**α**	**δ**	**γ**
None		6	2	2
Naringenin	2	1	0.4	0.7
Hesperetin	2	2	3	0.1
Tangeretin	0.8	0.4	0.04	0.02
Nobiletin	0.4	2	0.8	0.8

TABLE 7.3
Synergistic Effects of 1:1 and 1:1:1 Combinations of Flavonoids, Tocotrienols, and Tamoxifen[a] on Inhibition of Proliferation of MDA-MB-435 ER– Human Breast Cancer Cells in Culture [IC_{50} (μg/ml)]

	Tocotrienols			
Flavonoid	**None**	**α**	**δ**	**γ**
None	90	2	6	2
Naringenin	10	6	2	0.5
Hesperetin	13	2	6	9
Tangeretin	0.5	0.1	0.1	0.01
Nobiletin	0.5	2	0.25	0.5

[a] Tamoxifen was present in each case in these assays. Thus, the top line gives results for 1:1 combinations of tocotrienols and tamoxifen, while the left-hand column gives results for 1:1 combinations of flavonoids with tamoxifen. All other results are for 1:1:1 combinations.

and tamoxifen, tangeretin + γ-tocotrienol + tamoxifen was the most effective in ER– cells (IC_{50} 0.01 μg/ml) (Table 7.3) and hesperetin + δ-tocotrienol + tamoxifen was the most effective in ER+ cells (IC_{50} 0.0005 μg/ml) (Table 7.4).[25]

TABLE 7.4
Synergistic Effects of 1:1 and 1:1:1 Combinations of Flavonoids, Tocotrienols, and Tamoxifen[a] on Inhibition of Proliferation of MCF-7 ER+ Human Breast Cancer Cells in Culture [IC_{50} (μg/ml)]

	Tocotrienols			
Flavonoid	**None**	**α**	**δ**	**γ**
None	0.4	0.1	0.003	0.01
Naringenin	1.2	0.1	0.4	0.008
Hesperetin	0.3	0.4	0.0005	0.8
Tangeretin	0.04	0.4	0.02	0.02
Nobiletin	0.4	0.07	0.001	0.09

[a] Tamoxifen was present in each case in these assays. Thus, the top line gives results for 1:1 combinations of tocotrienols and tamoxifen, while the left-hand column gives results for 1:1 combinations of flavonoids with tamoxifen. All other results are for 1:1:1 combinations.

B. Animal Studies

1. Chemically Induced Mammary Carcinogenesis Model

The ability of citrus juices and flavonoids to inhibit mammary tumors induced in female Sprague-Dawley rats by 7,12-dimethylbenz[a]anthracene (DMBA) was investigated.[11] Rats were fed a semipurified diet contaning 5% corn oil. One group was given double-strength (reconstituted from frozen concentrate at two times normal strength) orange juice and another double-strength grapefruit juice in place of drinking water. For these groups, the carbohydrate component of the diet was reduced to compensate for the carbohydrate component in the fruit juices. A third group was given naringin and a fourth group was given naringenin, each mixed in the food in amounts comparable to those obtained by drinking the double-stength grapefruit juice. A fifth group was fed the semipurified diet and served as a control. The rats were palpated for tumors weekly. After 16 weeks, they were sacrificed and the tumors excised, weighed, and sent for histological examination.

Double-strength orange juice inhibited tumorigenesis more effectively than double-strength grapefruit juice (Figure 7.4), although the flavonoids present in these juices were equally effective *in vitro*.[11] This indicates that hesperetin probably retains its effectiveness *in vivo* better than naringenin, because the respective compounds are present in each juice at similar levels. It is noteworthy that although orange juice concentrate appeared to inhibit mammary carcinogenesis, the rats in this group showed larger weight gains than those in other groups.[11] The smaller tumor burden in rats given orange juice does not therefore appear to be due to nonspecific growth inhibition. Naringin (glycoside form of the flavonoid naringenin from grapefruit) also inhibited mammary carcinogenesis (Figure 7.4), but the rats in this group showed the least weight gains than those in other groups, and this may have had an influence on carcinogenesis.[11] When the experiment was repeated using a semipurified diet containing 20%, instead of 5%, corn oil, similar results were obtained.[11]

2. Mammary Xenograft Model

It is well known that estrogen receptor-negative MDA-MB-435 human breast cancer cells will develop into tumors and metastasize to the lung when injected into the mammary fat pads of immunodeficient mice. This animal model provides a more direct link to our *in vitro* studies and will allow us to study possible effects related to absorption, distribution, and metabolism of citrus

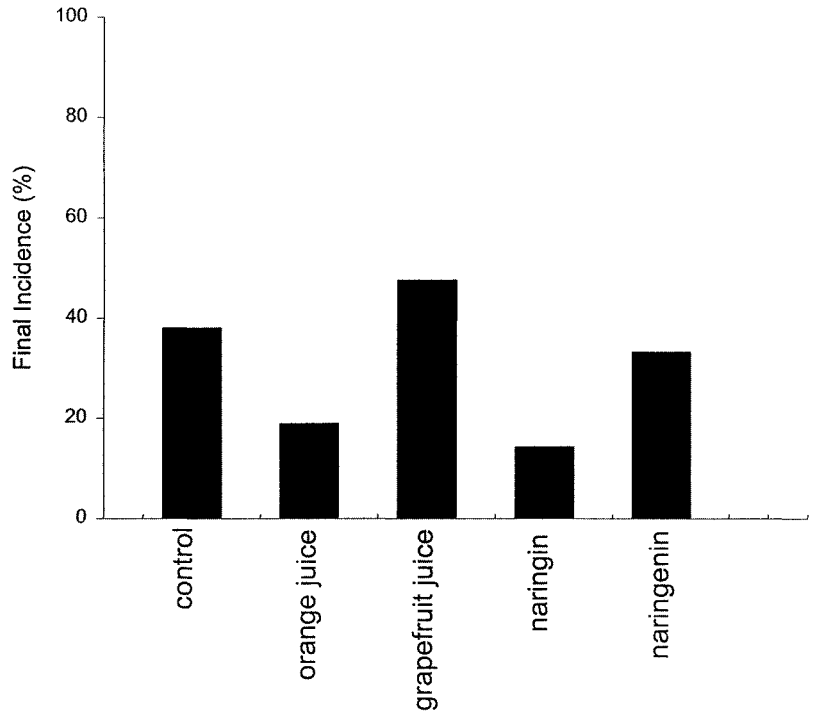


FIGURE 7.4 Final incidence of mammary tumors in female Sprague-Dawley rats induced with DMBA.

juices and their constituents on their ability to inhibit growth and metastasis of the human breast cancer cells.

A study was therefore conducted to determine the effects of orange juice, grapefruit juice, and their constituent flavonoids, on the growth of MDA-MB-435 human breast cancer cells injected into the mammary fat pads of nude mice.[26,27] The animals were randomly divided into seven groups of 24 animals each. They were fed a semipurified diet containing 5% corn oil. One group was given double-strength orange juice and another double-strength grapefruit juice instead of drinking water. For these groups, the carbohydrate component of the semipurified diets was reduced to compensate for the carbohydrate in the fruit juices. A third group was given naringin, a fourth naringenin, a fifth group hesperidin, and a sixth group hesperetin, each mixed in the diet to provide amounts comparable to those obtained by drinking the double-strength juices. A seventh group was fed the semipurified diet with plain drinking water and served as a control. After 1 week, the mice were anaesthesized with metofane and 1×10^6 MDA-MB-435 ER– human breast cells were injected in a volume of 50 μl into a right-sided mammary fat pad, which was exposed by a 5-mm incision. This was to ensure that the cells were injected into the mammary fat pad and not into subcutaneous space. The mice were weighed and the inoculation site was palpated for tumors at weekly intervals. The palpable mammary fat pad tumors were measured weekly, using callipers. After 11 weeks, the animals were sacrificed and the tumors, lymph nodes, and lungs were excised, weighed, and sent for histological examination.

The incidence of mammary fat pad tumors was reduced by more than 50% in the mice given orange juice, grapefruit juice, or naringin as well as hesperidin and naringenin (Figure 7.5).[27] Lymph node metastases and lung metastases were lowest in the orange juice and grapefruit juice groups, followed by the groups given naringin, hesperidin, or naringenin.[27] Our results indicate that growth and metastasis of these tumors in nude mice are strongly inhibited by orange and grapefruit juice and this inhibition cannot be entirely attributed to their constituent flavonoids. We have also investigated another class of compounds present in citrus, the limonoids, which have anticancer activity.

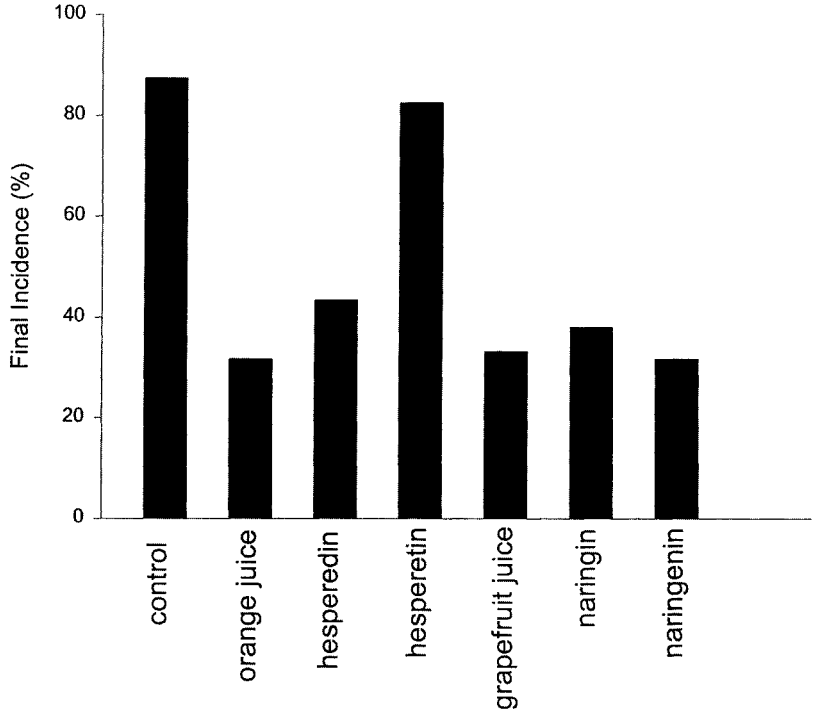


FIGURE 7.5 Final incidence of mammary fat pad tumors in female immunodeficient mice after injection of MDA-MB-435 human breast cancer cells into the mammary fat pad.

Citrus limonoids are one of the two bitter principles found in citrus fruits, such as lemon, lime, orange, and grapefruit.[28] They have been shown to have anticancer activity.[28] Nomilin reduced the incidence and number of chemically induced forestomach tumors in mice when given by gavage.[29] The addition of nomilin and limonin to the diet inhibited lung tumor formation in mice and topical application of the limonoids was found to inhibit both the initiation and the promotion phases of carcinogenesis in the skin of mice.[30] We have recently tested the effect of nomilin, limonin, and limonin glucoside on the proliferation and growth of ER– human breast cancer cells in culture. Nomilin was the most effective, having an IC_{50} of 0.4 μg/ml. We also tested a glucoside mixture and found it to have an even lower IC_{50} of 0.08 μg/ml.[31]

III. CITRUS FLAVONOIDS AND HYPERCHOLESTEROLEMIA

Elevated levels of blood cholesterol are known to be among the major risk factors associated with coronary heart disease (CHD), the leading cause of death in North America. The association is largely due to the importance of cholesterol, especially low-density lipoprotein (LDL) cholesterol, in the formation and development of atherosclerotic plaque, the underlying pathological condition of CHD. Blood concentrations of total and LDL cholesterol are influenced by diet, and dietary intervention has been widely used in prevention and treatment of hypercholesterolemia. Dietary strategies commonly used to reduce LDL cholesterol levels include changes in the intake of several macro- and micronutrients including fat, cholesterol, carbohydrates, and protein.[32] During recent years, a number of reports suggested that another possible way of improving blood lipid profile could be via increased intake of flavonoids.[33]

Previous epidemiological studies showed that consumption of fruit and vegetables is associated with reduced risk of cardiovascular disease[34] and the beneficial responses were postulated to be

due to flavonoids.[33] Cardioprotective effects of flavonoids appear to be largely related to their action as antioxidants and as inhibitors of platelet aggregation.[33] However, some of the flavonoid preparations were also reported to produce cholesterol-lowering responses in animals and in cells.[13,15,35-37] Among the plant flavonoids previously investigated for their possible cholesterol-lowering potential, the best known are isoflavones from soybean, consisting mainly of genistein. Dietary soybean isoflavones caused decreases in VLDL (very low-density lipoprotein) and LDL cholesterol in some animal models;[38,39] however, these beneficial changes were not confirmed in other animal and human studies.[40-42] The principal citrus flavonoids, hesperetin from oranges and naringenin from grapefruit, are structurally similar to genistein. Hesperidin and a mixture of flavonoids containing mainly hesperidin and naringin were also reported to produce hypolipidemic effects in cholesterol-fed rats.[13-15] This suggested that citrus flavonoids, and the juices from which they originate, could have cholesterol-lowering potential.

A. Animal Studies

To determine whether dietary citrus juices could produce cholesterol-lowering responses *in vivo*, we investigated their effects in rabbits in which hypercholesterolemia associated with an elevation of LDL cholesterol was induced by feeding cholesterol-free, casein-based, semipurified diet.[43] In this study, replacing drinking water with either double-strength orange juice or double-strength grapefruit juice reduced elevated levels of LDL cholesterol by 43 and 32%, respectively[44] (Table 7.5). This was associated with a significant, 42% reduction of liver cholesterol esters but not with increases in fecal excretion of cholesterol and bile acids. The decreases in LDL cholesterol were unlikely to be due to the additional intake of sugars from the juices, since this was compensated by modifications in the composition of semipurified diets and since, in rabbits, sugars were reported to have little effect on hypercholesterolemia.[45] The juices were also unlikely to act as cholesterol sequestrants in the intestine, since they did not increase fecal excretion of cholesterol and its products. Finally, the cholesterol-lowering effects of the juices were unlikely to be related to their high content of vitamin C since this is not a required nutrient in the rabbit.[46] Therefore, our data allowed us to speculate that changes in LDL cholesterol and in the liver cholesterol esters might be induced by minor components of the juices, possibly flavonoids. In support, recent studies showed that dietary supplementation with mixtures of citrus flavonoids containing largely hesperidin and naringin lowered serum cholesterol in rats fed a cholesterol-enriched diet.[15] This effect was associated with reduced *in vitro* activity of acyl CoA:cholesterol *O*-acyltransferase (ACAT), an enzyme responsible for cholesterol esterification in the liver.

TABLE 7.5
Effects of Dietary Orange Juice and Grapefruit Juice on Serum Total and LDL Cholesterol Levels in Rabbits with Experimental Hypercholesterolemia

		Cholesterol mmol/l	
Group	N	Total	LDL
Control	12	4.90 ± 0.47	2.91 ± 0.32[a]
Orange juice	11	3.55 ± 0.49	1.66 ± 0.29[b]
Grapefruit juice	11	3.85 ± 0.35	1.99 ± 0.22[b]

Values are means ± SEM. Values bearing different letters are significantly different at $P < 0.05$.

B. Cell Culture Studies

To determine whether citrus flavonoids could alter cholesterol metabolism directly in the liver, we investigated their mechanism of action in human liver cell line HepG2.[47] This model has been used because HepG2 cells can secrete as well as catabolize lipoproteins similar to LDL.[48] In these experiments, confluent HepG2 cells were preincubated for 24 h in serum-free medium which inhibits cell proliferation and stimulates biosynthesis of cholesterol-containing lipoproteins. They were subsequently exposed to a range of nontoxic concentrations (as determined by MTT viability assay)[22] of either hesperitin or naringenin for another 24 h. At the end of the incubation, changes in medium levels of apolipoprotein B (apo B), the structural protein of LDL, were evaluated by ELISA and compared to changes induced in the absence of flavonoids.[47] The results showed that both hesperetin and naringenin caused a similar, dose-dependent reduction of net apo B secretion (Figure 7.6), consistently with LDL cholesterol-lowering responses observed in rabbits given citrus juices.[44] Since at the concentration 60 μg/ml, both flavonoids reduced medium apo B very effectively (by 76 and 81%, for hesperetin and naringenin, respectively), these concentrations were used to characterize apo B responses further and to investigate underlying mechanisms. The results of our studies demonstrated that both flavonoids caused an approximate 50% medium apo B reduction after 4 h of the incubation and this was not associated with changes in the incorporation of ^{3}H-leucine into total cellular and secreted proteins over the same period. This indicated that the apo B responses to flavonoids were rapid and selective, most likely due to a post-translational modulation of apo B secretion.[47] Our further studies on the possible mechanisms of this modulation revealed that the apo B–lowering effect of flavonoids was maintained in the presence of a specific inhibitor of proteases responsible for the intracellular apo B degradation. However, the effect

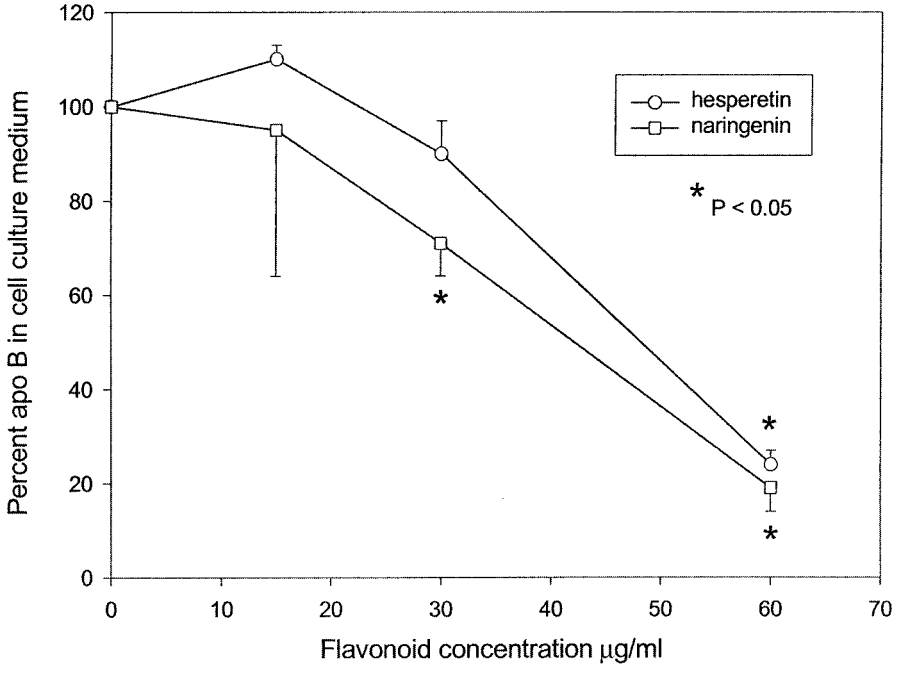


FIGURE 7.6 Effects of various nontoxic concentrations of hesperetin and naringenin on apo B accumulation in the media of HepG2 cells. Cells were incubated for 24 h in the presence of 0 to 60 μg/ml of either hesperetin or naringenin. Medium apo B concentrations were measured by ELISA and expressed per milligram cell protein. Values are means ± SEM, n = 3.

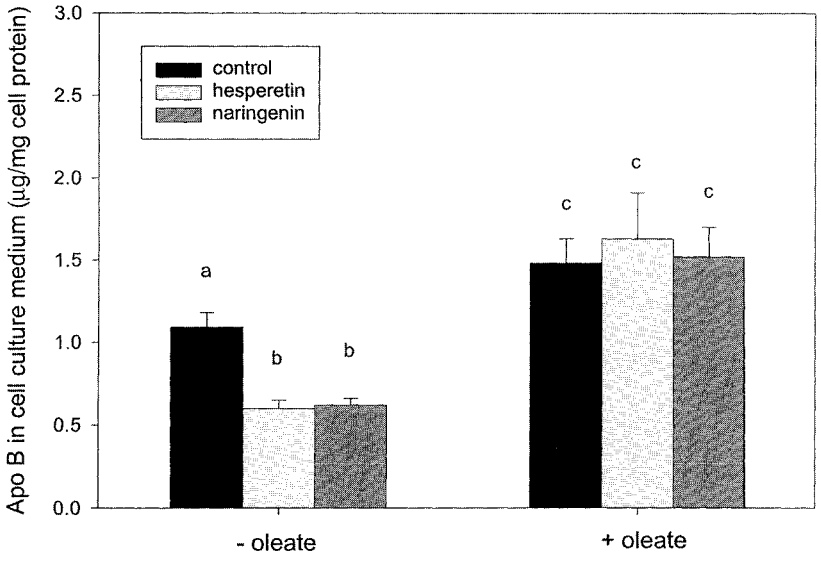


FIGURE 7.7 Effects of citrus flavonoids on apo B concentration in medium of HepG2 cells in presence of oleate. Cells were preincubated for 1 h with or without 0.8 m*M* sodium oleate and then incubated for 4 h in the absence or presence of flavonoid (60 µg/ml), and also in the absence or presence of oleate. Medium apo B concentrations were measured by ELISA and expressed per mg cell protein. Values are means ± SEM, $n = 4$. Values with different letters are significantly different, $P < 0.05$.

disappeared during the coincubation of cells with oleate, a compound known to stimulate cellular biosynthesis of neutral lipids[47] (Figure 7.7). This indicated that flavonoids were not likely to exert their apo B–lowering action by increasing the intracellular apo B degradation during early stages of lipoprotein formation. Instead, they appeared to interfere with the availability of neutral lipids required for the assembly and secretion of lipoproteins.[47] In agreement, our ^{14}C-acetate labeling study showed a 50% decrease in cholesteryl ester synthesis in cells exposed for 4 h to either hesperetin or naringenin.[47] A similar observation has been reported by our collaborators, who demonstrated that naringenin has ability to suppress the *in vitro* activity of hepatic ACAT.[49]

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8 Flavonoids as Antioxidants

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I. GENERAL BACKGROUND ON PHYTOCHEMICALS AS ANTIOXIDANTS

The oxidant reactions of free radicals, molecules with unpaired electrons, are thought to contribute to many health problems (reviewed in Reference 1). Antioxidants are agents which restrict the deleterious effects of oxidant reactions. These effects can be direct (i.e., eliminating certain free radicals) or indirect (i.e., preventing radical formation). The body produces certain endogenous antioxidants (i.e., certain enzymes) and antioxidants can be consumed in the diet. Some dietary antioxidants, such as vitamin E, are essential nutrients (the body cannot function normally without them). The diet also contains other components potentially capable of antioxidant actions. These belong to the class of food components known as phytochemicals. These compounds, such as the flavonoids, are not absolutely required parts of the diet, but may confer health benefits such as antioxidant actions.[2] It might be said that one can live without phytochemicals, but one might live better with them.

If phytochemicals exert antioxidant actions in people, then body antioxidant capacities should diminish with a low phytochemical diet. This idea has been tested to a limited degree in a study from our laboratory.[3] Patients undergoing renal dialysis consumed one of three semipurified, liquid formulas, as a sole nutrition source for 3 weeks. Each formula contained the essential nutrients in amounts generally considered adequate, but did not contain major sources of phytochemicals. Nearly all of the subjects (n = 20 for each of the three formulas) showed depressed values for plasma total antioxidant status (TAS). The fall in TAS values could not be attributed to three major influences on TAS, namely, vitamin E, vitamin C, or uric acid. β-Carotene was also not a factor. Thus, this study provided some evidence that phytochemicals can influence antioxidant capacities.

Flavonoids are one of the major classes of phytochemicals.[4] Many flavonoid compounds may be capable of exerting antioxidant effects in humans.[4] These compounds, which are a family of polyphenols, are found in a variety of foods of plant origin. There is also the potential to use such compounds as pharmacological agents, an idea that has already been tested to a limited degree, both for naturally occurring flavonoids and synthetic compounds.[5,6]

Health-related research on flavonoids is actually not new, but widespread research on the biological actions of flavonoids has accelerated only recently. A personal note can be mentioned

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along these lines. In 1983, I coauthored a paper on interactions between a copper enzyme and the flavonoid catechin.[7] At that time, a number of researchers discouraged me from pursuing further work on flavonoids because "there is little interest in these compounds." Indeed, this past attitude can be confirmed by a Medline search of catechin for 1983. Only eight other papers are found. In contrast, a similar Medline search for catechin for 1998 reveals 83 papers.

At present, the practical implications for flavonoids in human health is still somewhat sketchy. However, the evidence that certain flavonoids exert health-promoting, antioxidant actions in humans is growing at a rapid pace. This chapter gives a brief overview of the possible mechanisms that could be associated with flavonoid antioxidant actions, summarizes some of the evidence that antioxidant actions actually occur in humans (including evidence that involves lipoprotein oxidation), discusses evidence for the operation of specific antioxidant mechanisms, and notes the concern about possible prooxidant effects of flavonoids. Many representative references are cited, but apologies are issued to the numerous authors who are not cited here, but who have contributed papers on flavonoids as antioxidants.

II. POSSIBLE ANTIOXIDANT MECHANISMS OF FLAVONOIDS

Often, flavonoid antioxidant effects are conceived only in terms of a single biochemical action, direct reactions with radicals. These direct reactions produce a so-called scavenging of free radicals, where the unpaired electron of a radical becomes paired without ultimately generating another radical.[1] However, potential antioxidant actions of flavonoids must be considered as multiple for two reasons. First, as listed in Table 8.1, there are at least six different possible antioxidant mechanisms for flavonoids. Second, the free radical–scavenging effects of flavonoids are not necessarily a single biochemical action.

Although Table 8.1 lists direct radical scavenging as a single mechanism, this action could involve more than one type of reaction within an oxidant process. An example of this oxidant process is given by Cook and Samman.[4] They state that there are three different stages of radical-mediated oxidation of membrane lipids:

1. Initiation (free radicals remove a hydrogen from a polyunsaturated fatty acid to form a lipid radical);
2. Propagation (the lipid radical plus molecular oxygen forms lipid peroxy radical, then breaks down to more radicals);
3. Termination (the new radicals react together or with antioxidants to eliminate radicals).

Cook and Samman[4] state that flavonoids could act at any of these stages. Flavonoids could block initiation by scavenging primary radicals such as superoxide. Flavonoids could also react with peroxy radicals to slow propagation. In addition, the flavonoid radical intermediates, formed after reacting with peroxy radicals, can react with the other radicals formed during propagation. This would accelerate the termination process.

TABLE 8.1
Possible Antioxidant Mechanisms of Flavonoids

A.	Direct radical scavenging
B.	Downregulation of radical production
C.	Elimination of radical precursors (i.e., hydrogen peroxide)
D.	Metal chelation
E.	Inhibition of xanthine oxidase
F.	Elevation of endogenous antioxidants

Five of the possible antioxidant mechanisms in Table 8.1 can involve, at least in part, prevention of the formation of free radicals (Table 8.1, Mechanisms B to F). In some senses, these mechanisms might be termed as indirect antioxidant actions. These mechanisms include downregulation of the production of superoxide radical and hydrogen peroxide, a precursor of free radicals.[8–11] This effect, at least in some situations, could be accomplished through downregulation of protein kinase C, which is thought to trigger secretion of superoxide and hydrogen peroxide.[12,13] This protein kinase C mediation could be particularly true for the soy constituent genistein, which belongs to the flavonoid class known as the isoflavones. Genistein is a classic inhibitor of protein kinase C *in vitro*.[12] Still, it is not likely that this is the only way in which flavonoids could inhibit production of superoxide and hydrogen peroxide. Another consideration in regard to hydrogen peroxide is that flavonoids may also directly react with this compound in a manner that eliminates the potential for radical formation (Table 8.1, Mechanism C).[14,15]

Another way flavonoids may prevent radical formation is by chelation of transition metals (Table 8.1, Mechanism D). Some transition metals, such as iron, can catalytically form reactive free radicals.[1] Many flavonoid structures have the chemical properties to chelate these metals in a state where radical generation is inhibited.[16,17] In addition to this action, metals and flavonoids may also, in some circumstances, form complexes which actually eliminate radicals.[18]

Flavonoids could also act as antioxidants by inhibiting prooxidant enzymes (Table 8.1, Mechanism E). The most prominent example would be inhibition of xanthine oxidase,[19–21] which can, in certain states, produce superoxide radical.[22] The pathological significance of superoxide production by xanthine oxidase has been debated, but could be important in some health problems such as cardiac reperfusion injury.[22]

There is another possible indirect antioxidant action of flavonoids. Some of these compounds may elevate body concentrations of endogenous antioxidants which themselves eliminate free radicals or their precursors. An example would be superoxide dismutase 1,[23] which eliminates superoxide radical inside cells.[1,24]

III. EVIDENCE THAT FLAVONOIDS ACT AS ANTIOXIDANTS

What is the evidence that flavonoid antioxidant effects actually occur to any great extent in humans? A lot of the evidence is based on what flavonoids can do *in vitro*. Of course, studies done in test tubes or on culture plates cannot perfectly predict what will happen *in vivo*. Nonetheless, observations made *in vitro* can give insights into what actions are possible *in vivo*. From this perspective, each of the potential antioxidant mechanisms of flavonoids mentioned in Table 8.1 has been noted *in vitro* (see Section V). In addition, work in experimental animals has been used to justify the claim that flavonoids can act as antioxidants. These studies generally fall into one of four categories noted in Table 8.2.

TABLE 8.2
Evidence for Flavonoid Antioxidant Actions in Experimental Animals

A.	Depressed accumulation for products of oxidant reactions (i.e., lipid peroxides)[25,26]
B.	Inhibition of a rise in oxidant product concentrations caused by an external stress (i.e., administration of a carcinogen or chemically induced inflammation)[27–29]
C.	Improved resistance to pathology in animal models for human diseases which are thought to involve oxidant stress (i.e., effects on tumor numbers or inflammatory swelling)[27,28]
D.	Elevation of the concentrations of endogenous antioxidants, or prevention of their depletion by an externally induced stress[23,29]
E.	Elevated measures of plasma or serum antioxidant capacities determined *ex vivo*[30,31]
F.	Reduction of spin trap-detectable radicals during an oxidant stress (very little current data)[32]
G.	Inhibited oxidant stress-induced chemiluminescence determined *in situ*[33]

Flavonoids were given by diet or bolus administration, as either isolated compounds or as part of a more general dietary intervention (i.e., green tea consumption). The references cited for A through E are representative, but are not meant to be exhaustive.

One difficulty in many of these experimental animal studies is distinguishing whether an antioxidant effect is a primary or secondary effect of the flavonoids. For example, for category C, if a carcinogen is given to a rat, flavonoids may inhibit tumor development through an antioxidant effect or through some other action. A example of the latter would be inhibition of cytochrome P-450-mediated carcinogen activation.[34] This would limit the ability of that carcinogen to produce tumors, but would not really be an antioxidant effect of the flavonoids. Even if oxidation products are measured, such as lipid peroxides, this still does not always distinguish an antioxidant effect from simple prevention of carcinogen activation. If the carcinogen is not activated well, there will be little oxidant stress due to that carcinogen.

Despite this conceptual problem, the wide variety of experimental designs used in these studies suggests that antioxidant actions of flavonoids explain at least some of the results. This contention is supported further by the flavonoid-induced increases in measures of serum antioxidant capacities determined *ex vivo*.[30,31] These measurements are defined as *ex vivo* because flavonoid intake is *in vivo*, but the ability of plasma or serum to scavenge radicals is determined *in vitro*. Although exact interpretation of these type measurements carries some uncertainties, the results at least indicate that ingested flavonoids can impact radical-scavenging potential in an intact animal. The effects can be rather dramatic if the experimental conditions are manipulated in certain ways. This can be illustrated by data in Table 8.3. Our laboratory fed a low-phytochemical American Institute of Nutrition (AIN) diet to rats for 45 days. Half the rats consumed drinking water spiked with the flavonoid catechin (Table 8.3). The difference in serum TAS between catechin treatment and no treatment was extremely large. In most studies of TAS or related measurments, a 20% difference between two groups is considered good. In contrast, here the difference was nearly 2.5-fold. Admittedly, this rat study was designed to give a large response. A high dose of catechin was used and the basic diet was intended to minimize the presence of phytochemicals in the diet. Nonetheless, this study showed that flavonoids have the potential to exert a major influence on TAS values.

TABLE 8.3
Chronic Catechin Feeding Effects on Plasma TAS in Rats

Treatment	TAS (mmoles/l)
Control	1.5 ± 0.3
Catechin	3.5 ± 0.3

Note: Rats were fed a semipurified AIN diet and given tap water either with or without dissolved catechin (2.1 g/l) for 45 days.

Human studies of flavonoids as antioxidants are still limited in scope, although many more studies will likely come forth shortly. Table 8.4 categorizes the current observations. Most of the studies done so far examine flavonoid-rich foods rather than isolated flavonoids.

The epidemiology studies (Observation A, Table 8.4), although very important from a practical standpoint, have the same inherent problem as studies of diseaselike symptoms in the animal models. Both type studies do not always distinguish flavonoid antioxidant effects from other flavonoid effects. Moreover, the human epidemiological studies do not necessarily distinguish a cause-and-effect relationship for flavonoids from a coincidental effect. For example, an apparent protective effect for flavonoid-rich foods could actually be attributable to the vitamin C content of the foods.

TABLE 8.4
Observations from Human Studies of Flavonoids as Antioxidants

A.	In epidemiological studies, inverse correlations between flavonoid consumption and incidence of diseases thought to involve oxidant stress[4,35–37]
B.	Depression of the concentrations of oxidant products such as lipid peroxides[38]
C.	Elevation of concentrations of endogenous antioxidants, or prevention of their depletion during oxidant stress[38]
D.	Elevated measures of plasma or serum antioxidant capacities determined *ex vivo*[39–41]
E.	Inhibition of exercise-induced muscle tissue breakdown and inflammation[40]
F.	Depression of lipoprotein oxidation rates assessed *ex vivo*[42–44]

Note: References are examples, not an exhaustive list. Most of these studies involve consumption of flavonoid-rich foods, not isolated flavonoids.

Another possibility is that consumption of flavonoid-rich foods occurs to the greatest degree in people who emphasize a generally healthy lifestyle. Thus, the general lifestyle, rather than the flavonoid consumption per se, may explain the results.

For human studies, observations of flavonoid effects on oxidation products (i.e., lipid peroxides) or endogenous antioxidant concentrations (Observations B and C, Table 8.4) are still very limited in terms of study numbers and scope of design. For example, on September 1, 1999, a Medline search of lipid peroxide (or peroxides), plus flavonoid or flavonoids, did not yield a single paper showing flavonoid intake depressing lipid peroxide values in humans *in vivo*. A few such papers were found using other search strategies, but the number was small and the scope limited. For instance, one study found a depression in plasma values for malondialdehyde (a lipid peroxide product) during a 1-week period of high consumption of certain juices.[39] However, the contribution of flavonoids to this result is uncertain. The results could be due to other factors such as vitamin C, or the indirect effects of juice consumption displacing other beverages in the subjects' diets. Certainly, more work is needed in this area.

As with experimental animal studies, flavonoid consumption by humans has been associated with increases in plasma or serum indices of antioxidant capacities determined *ex vivo* (Observation D, Table 8.4). This indicates that in humans consuming various diets, flavonoid intake can influence overall radical-scavenging capacities of plasma or serum. However, as with the experimental animal studies, the potential impact on health of these effects cannot yet be assessed in a quantitative fashion.

One approach to evaluating possible antioxidant actions, including those of flavonoids, is not done as readily in humans as it is for experimental animals. This approach is to examine antioxidant intake effects on an acute oxidant stress in otherwise healthy subjects. In experimental animals, this can be done in various ways including injection of hepatotoxins such as carbon tetrachloride, administration of endotoxin, induction of an inflammatory condition, or exposure to hyperoxia. Obviously, ethical considerations limit such studies in humans. One alternative can be a bout of exercise. Although exercise is generally considered health promoting, it does precipitate an oxidant stress.[45] This stress is thought to contribute to the muscle tissue breakdown and inflammation that occurs during exercise.[45] Therefore, a bout of exercise can be one way of studying acute oxidant stress in otherwise healthy people. Not only do such studies provide basic insights into whether or not a food component exerts antioxidant effects, but these studies may also have some practical value for health promotion. The oxidant stress of exercise is thought to contribute to fatigue, muscle soreness, and risk of injury.[45–49] In addition, very strenuous exercise may increase cancer risk in the case of ultraendurance athletes, or in people who train hard, but sporadically (the "weekend warrior").[49]

Our laboratory[40] has carried out a pilot study in young men that simulated the "weekend warrior" behavior (Observation E, Table 8.4). The subjects, who were not highly trained aerobically, consumed soy protein beverage or whey protein placebo twice a day for 3 weeks. The soy protein beverage was rich in a category of flavonoids known as the isoflavones, while the whey did not contain such compounds. Before and after this 3-week consumption period, the subjects performed an exhaustive bout of aerobic exercise. The soy, but not the whey, group showed less muscle tissue breakdown and inflammation after the second exercise bout. Our laboratory plans to carry out more in-depth studies on soy and exercise. However, it should be noted that any effects of soy protein products may not be due to just the isoflavone contents.

IV. FLAVONOIDS AND LIPOPROTEIN OXIDATION

Lipoprotein oxidation has become a popular means of studying possible antioxidant effects. One reason is that fairly short term dietary interventions can produce changes in lipoprotein oxidation values.[42,50] Another reason is that lipoprotein oxidation is considered highly relevant to a major human health problem, atherolsclerosis.[51] In human studies, the oxidation is studied *ex vivo* (the oxidation is initiated after removal of the lipoproteins from the subjects). However, variations in the donors' lipoprotein, such as variations in preformed lipid hydroperoxide contents, is assumed to influence the oxidation data.[51] Generally, these studies examine oxidation of low-density lipoprotein (LDL), but some studies examine the combination of LDL plus very low density lipoproteins (VLDL).

Lipoprotein oxidation seems particularly useful for study of flavonoids. For one thing, both lipoprotein oxidation and flavonoid intake are thought to be relevant to cardiovascular disease.[35,51] Another issue is that many flavonoids are potent inhibitors of lipoprotein oxidation when added to the lipoprotein *in vitro*.[52–56] The studies done *in vitro* have initiated the oxidation in a variety of ways (metal ions, organic chemical reactions, and cell initiated events).

The results for studies on flavonoid intake and lipoprotein oxidation have varied greatly. Some have found an effect,[42–44] and some have not.[41,53,57] Some of this variability in results is likely attributable to differences in the study design. These differences are noted in Table 8.5. The first variable on the list, different types of flavonoids, could be very important, but does not seem to be the whole answer. For instance, one study of tea flavonoids has found effects on LDL oxidation,[43] while others have not.[41,57]

Variable B, Table 8.5, whole food vs. flavonoid concentrate, has produced two hypotheses. One, the combination of whole-food ingredients may be more effective than just a single flavonoid, or even just the flavonoid fraction of a food (i.e., vitamin C plus flavonoids may be more effective than either alone). Two, the absorption and metabolism of the flavonoids may depend on other food components. For example, the alcohol in red wine may make flavonoids more biologically active than they would be without the alcohol.[59]

TABLE 8.5
Design Variable for Studies of Flavonoid Intake Effects on Lipoprotein Oxidation

A.	Type of flavonoids
B.	Whole foods vs. flavonoid concentrates
C.	Lipoprotein isolation method
D.	Length of flavonoid ingestion period
E.	Type of subjects
F.	Placebo control vs. no placebo control
G.	Background diet of the test subjects

The next variable, background diet of the subjects, may be important in either enhancing or restricting a flavonoid intervention effect. For instance, a very low background flavonoid intake might make any flavonoid effects more pronounced. However, a low background intake does not guarantee a flavonoid effect on lipoprotein oxidation. Our laboratory tested this idea in rats, where dietary conditions are easier to control than with humans. In this study, a low-flavonoid, semipurified diet was fed for 45 days, with half the rats ingesting catechin in the drinking water at 2.1 g/l (unpublished study). Despite the catechin producing a large rise in TAS, there was no effect on LDL + VLDL oxidation lag time or propagation rate.

One question raised about the whole area of flavonoids and lipoprotein oxidation asks, What minimal plasma concentration of flavonoids are needed to affect lipoprotein oxidation? Obviously, the answer may vary with the type of flavonoids. However, even aside from this issue, this question is not yet easy to answer. One problem is that much of the methodology for measuring flavonoids and their metabolites in plasma is just emerging. Despite this, it has already been contended that the flavonoid concentrations often used to study lipoprotein oxidation *in vitro* are too high to apply to most human situations.[41]

This may be true in some cases, but not in others. Our laboratory considered this issue for a high dose, quercetin-containing, citrus flavonoid complex supplementation in women with Type II diabetes.[53] A 3-week treatment did not alter LDL + VLDL oxidation lag times nor propagation rates. A crude assessment of quercetin-like compounds in plasma indicates that the supplementation can produce plasma levels similar to those that strongly inhibit copper ion-induced oxidation of LDL + VLDL *in vitro*. In contrast, in another study, tea consumption does affect LDL oxidation *ex vivo* despite lower plasma levels of catechin flavonoids than those inhibiting LDL oxidation *in vitro*.[43] Thus, differences between flavonoid concentrations in plasma and those used for data gathered *in vitro* do not explain all the different results for lipoprotein oxidation studies.

Another issue is how much of the flavonoids present in plasma, following oral ingestion, remain with the lipoproteins upon isolation prior to initiating oxidation *in vitro*. Obviously, when flavonoids are added directly to isolated lipoproteins to study oxidation *in vitro*, all the added flavonoids are present during the oxidation assessments. On the other hand, this is not necessarily true for orally ingested flavonoids. Thus, different flavonoids may partition differently during lipoprotein oxidation. This could be very important in determining how ingestion of different types of flavonoids affects lipoprotein oxidation results.

However, this may not always be the case. Ingested flavonoids may not always have to remain with lipoproteins after isolation to affect oxidation data obtained *ex vivo*. An important consideration here is that preexisting lipid hydroperoxide in the lipoprotein affects oxidation measured *ex vivo*.[51] This variable would depend on processes occurring *in vivo*. These processes could be influenced by an antioxidant that does not isolate with lipoprotein after blood is drawn. For example, flavonoids could scavenge free radicals *in vivo* before they reach the lipoproteins to produce lipid hydroperoxide. The flavonoids could also downregulate phagocyte secretion of radicals which produce lipid hydroperoxide in lipoproteins. Some data directly support the idea that flavonoids do not always have to remain with isolated lipoproteins to affect the oxidation data obtained *ex vivo*. A prime example is that soy isoflavone intake inhibits LDL oxidation *ex vivo* despite low isoflavone contents in the isolated LDL.[44]

V. EVIDENCE FOR SPECIFIC ANTIOXIDANT MECHANISMS OF FLAVONOIDS

Even as evidence is accumulating that flavonoids can act as antioxidants, it is not always easy to decide which particular mechanisms from Table 8.1 are important *in vivo*. It is very possible that different mechanisms are involved to varying extents for different flavonoid effects.

It is beyond argument that the first mechanism from Table 8.1, free radical scavenging, has been well demonstrated *in vitro*. This has been done with a great variety of experimental conditions. For example, various flavonoids have produced antioxidant actions against radicals generated and

detected in a number of ways.[4,16,18,54,60–67] Generation methods have included enzyme systems, nonenzymatic organic chemical reactions, and metal-catalyzed events. Detection methods include direct electron spin resonance (ESR) measurements of free radical disappearance, injury to cultured cells and cell organelles, diminished generation of oxidant products, and inhibition of oxidation of target molecules such as lipids, lipoproteins, liposomes, or DNA. The sheer volume of data in this area presents a strong case that flavonoids are capable of direct radical scavenging. However, data gathered *in vitro* have not settled three crucial questions:

1. Do flavonoid concentrations *in vivo* reach and maintain values where the scavenging would have a major impact on antioxidant defenses?
2. If the answer to the first question is yes, then in what body sites does this occur?
3. Does metabolic modification of flavonoids diminish their scavenging potential?

For the first two questions, the above-noted increases in plasma antioxidant capacities following flavonoid ingestion support the idea that radical scavenging by flavonoids is possible in plasma *in vivo*. However, it remains to be determined how much this action actually occurs and how much these actions impact health. One need in this research is a search for oxidation products of flavonoids *in vivo*. If flavonoids scavenge radicals, then certain flavonoid products will be formed. Initially, these products could be identified after reactions carried out *in vitro*. Then, it can be determined whether these same products can be found *in vivo*. If they are found, then this would be good evidence that flavonoids scavenge radicals *in vivo*. For the last question, the activity of metabolites, an initial indication of a "yes" answer seems to be true for at least some metabolites of quercetin.[68] Still, a lot of work remains to be done about flavonoid scavenging effects.

Another possible antioxidant action of flavonoids is the ability to suppress cellular production of radicals and their precursors such as hydrogen peroxide. This has been demonstrated *in vitro* using isolated phagocytes or cell organelles.[8–12] However, this action is harder to assess *in vivo*. One relevant indirect measurement can be measurement of plasma myeloperoxidase during oxidant stress. Since secretion of myeloperoxidase by certain phagocytes can occur at the same time as secretion of superoxide and hydrogen peroxide,[69] increases in myeloperoxidase can sometimes be construed to indicate elevated superoxide and peroxide production *in vivo*. In the previously mentioned study of soy and exercise,[40] soy isoflavone intake was shown to reduce exercise-induced increases in myeloperoxidase. Future work is still needed to explore more fully the relationship of flavonoids with secretion of radicals and their precursors.

Many flavonoids have chemical structures that allow chelating of transition metals, such as iron and copper,[4,16–18,62] an action that could inhibit metal-catalyzed formation of free radicals. This inhibition has been demonstrated *in vitro*, mostly indirectly by examining oxidant damage to a target molecule.[4,16–18,62] In addition, some flavonoids have been shown to protect against iron-induced damage to cultured cells.[64,70,71] There have also been some studies showing that flavonoids can block iron-induced injury in rodents.[32,72] In one of these studies, certain flavonoids actually diminish the concentration of radicals detectable by ESR spin trapping.[32] However, none of these results in themselves demonstrates that flavonoids actually prevent iron from forming free radicals. Alternatively, these results could be simply due to flavonoids eliminating the radicals after they are formed. On the other hand, there is one observation that suggests that iron chelation is at least partially involved in protection against iron-overload injury. In cultured rat hepatocytes, three flavonoids show cytoprotection effects in proportion to their iron chelating ability.[70]

Flavonoid interactions with metals could have an additional antioxidant effect other than inhibiting metal-catalyzed radical formation. Flavonoid–metal complexes can be a catalyst that actually eliminates radicals. An example of this has been demonstrated *in vitro* with a rutin–copper complex.[18] The complex is much more potent than rutin alone in eliminating superoxide radical and preventing lipid peroxidation in rat liver microsomes. Other flavonoids have not been tested

to see whether their copper complexes can act as antioxidant catalysts. However, the general idea that flavonoid–copper complexes can act catalytically has a precedent. Copper–catechin complexes can catalytically act like the copper enzyme lysyl oxidase, which functions in connective tissue maturation.[7] It remains to be seen whether copper–flavonoid complexes can act as catalysts *in vivo*.

Another possible flavonoid antioxidant mechanism is elimination of radical precursors such as hydrogen peroxide. A few studies done *in vitro* have shown that certain flavonoids can block hydrogen peroxide–induced oxidation of biomolecules or cell cytotoxicity.[15,73] However, these studies do not distinguish whether flavonoids eliminate hydrogen peroxide or just react with products of hydrogen peroxide. In other words, flavonoids may protect biomolecules or cells by eliminating radicals after they are formed from hydrogen peroxide. However, in the cell cytotoxicity studies,[73] the flavonoids could not be replaced with vitamin E or ferrulic acid, which are more general antioxidants. Moreover, there are at least two reports showing that flavonoids can react directly with hydrogen peroxide.[14,74] It is not yet certain whether such reactions can account for flavonoid antioxidant effects against hydrogen peroxide *in vitro*.

As noted above, flavonoids could exert antioxidant actions by inhibiting the activities of the prooxidant enzyme xanthine oxidase. Certain flavonoids definitely do this *in vitro*.[19–21,75] However, the importance of this observation remains unclear. One concern is that the flavonoid concentrations used to inhibit xanthine oxidase *in vitro* vary considerably. Some of these concentrations could be fairly high compared to what might be seen *in vivo*. However, this judgment is currently hard to make. There are just a few studies of plasma concentrations of flavonoids, and these generally focus on just ingested flavonoid. Moreover, these studies usually do not account for metabolites of the originally ingested flavonoid. In addition, these plasma studies do not give any insight into possibly high local concentrations in certain tissues.

Another issue with current xanthine oxidase inhibition data is how the inhibition is accomplished. Flavonoids can precipitate proteins *in vitro*,[76] which could explain the inhibition of xanthine oxidase activity *in vitro*. However, this same precipitation may not occur *in vivo*. In the latter situation, flavonoids may bind nonspecifically to many macromolecules. In that case, the amount binding to any one particular macromolecule may not be enough to cause a precipitation. One study does provide some evidence that the presence of proteins other than xanthine oxidase does not totally preclude flavonoid inhibition of xanthine oxidase *in vitro*.[75] In this work, albumin addition only partially prevents xanthine oxidase inhibition by certain flavonoids. Although this is a useful observation, more work is needed to establish the importance of flavonoid inhibition of xanthine oxidase *in vivo*.

A slightly different issue can be raised in terms of flavonoid effects on xanthine oxidase. This enzyme can exist in two forms: a dehydrogenase form that does not generate superoxide radical and an oxidase form that does.[22] Conversion to the oxidase form is thought to be a contributor to certain health problems such as reperfusion injuries.[22] One study has shown that in rats, some flavonoids inhibit conversion to the oxidase form during renal reperfusion.[77] It is hoped that this interesting observation will spur other work in this area.

Flavonoids can also act as antioxidants by elevating body contents of endogenous antioxidants or by preventing their depletion by certain stress states. The extent to which flavonoids can affect endogenous antioxidant concentrations is not yet characterized. This is true from a number of perspectives including the range of flavonoids that can exert these effects, a dose–response relationship for the effects, the range of body sites that can be affected, and the circumstances under which the effects will occur. One example of a flavonoid–endogenous antioxidant interaction involves the antioxidant enzyme superoxide dismutase 1. A published paper[23] indicates that in mice, inclusion of green tea in the drinking water increases colon levels of superoxide dismutase 1, which is also called Cu–Zn SOD. This increase is reportedly based on immunohistochemical staining. Our laboratory has obtained a similar result for Cu-Zn SOD activities in rats using a synthetic version of the green tea flavonoid catechin (Figure 8.1). Interestingly, this effect was seen when

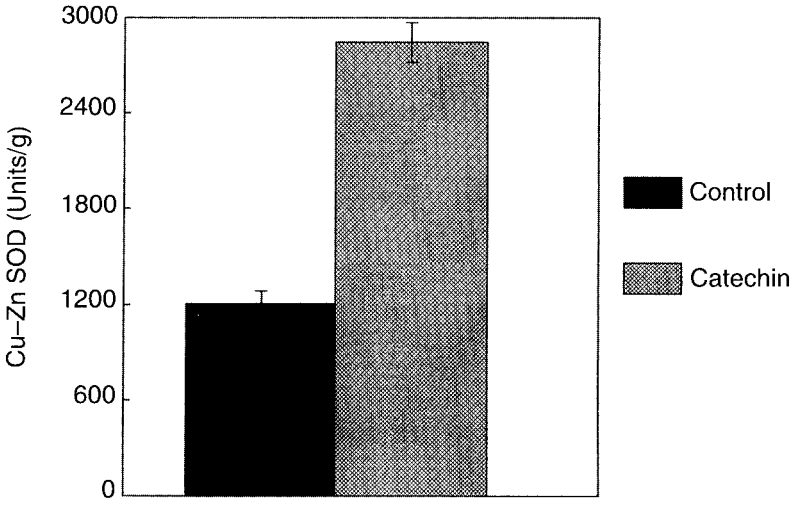

FIGURE 8.1 Colon Cu-Zn SOD in rats given catechin. Rats were fed a standard, mixed-feed diet and given tap water either with or without dissolved catechin (2.1 g/l) for 45 days.

rats were fed a mixed-feed type of diet, but not with a semipurified diet. When the effect was seen, it was tissue specific. In the pancreas, a smaller response was seen compared with the colon, and no response was seen in the liver or lung (unpublished data). In another study,[78] little to no effect on liver SOD activities is reported for feeding any of three flavonoids to rats. This lack of effect occurs despite a depression of liver lipid peroxidation. Similarly, bolus quercetin treatment restricts ethanol-induced gastric mucosal injury without changing mucosal SOD activities.[79]

The flavonoid silymarin has been reported to increase lymphocyte SOD 1 activities. A series of studies have been done with silymarin in regard to Cu-Zn SOD in erythrocytes and lymphocytes from patients with alcoholic cirrhosis in Hungary.[80] Silymarin consumption raises initially low Cu-Zn SOD activity and protein values in lymphocytes and erythrocytes. The same general effect is also achieved by incubating silymarin with these same cell types *in vitro*. However, data are lacking on silymarin actions *in vivo* in other types of people (i.e., healthy subjects). Possibly, the effects in the patients with cirrhosis represent a general protection of cell protein integrity rather than a specific induction of SOD 1.

Some flavonoids may be able to prevent inactivation of SOD 1 in a given body site during an oxidant stress, but not have any effect on activities under normal circumstances. An example of this is seen for catechin and rat lung SOD.[29] This study reports that bolus catechin administration prevents a fall in lung SOD activities in rats treated with the oxidant stimulant diethylmaleate, but does not affect prestress SOD values.

Flavonoids can also affect concentrations of the antioxidant glutathione. In rodent studies, there is considerable specificity for flavonoid type and tissue. A number of different flavonoids are reported either to increase gastrointestinal glutathione concentrations or to prevent their depletion by inflammatory stress.[81–83] Some other rodent tissues also respond to flavonoid ingestion with either increased glutathione contents or protection against stress-induced depletion.[29,84,85] However, one study[86] indicates that the flavonoid silymarin does not increase glutathione contents of the kidney, lung, or spleen despite increasing levels in intestine, liver, and stomach. In contrast, our laboratory has found that chronic ingestion of synthetic catechin has no effect on liver glutathione contents but nearly doubles these values in lung (unpublished results). This lung effect seems to have protective consequences. As shown in Figure 8.2, catechin ingestion partially protected against lung lipid peroxidation caused by diethylmaleate, a chemical that depletes lung glutathione.[87] In contrast, in mice, administration of two flavonoids

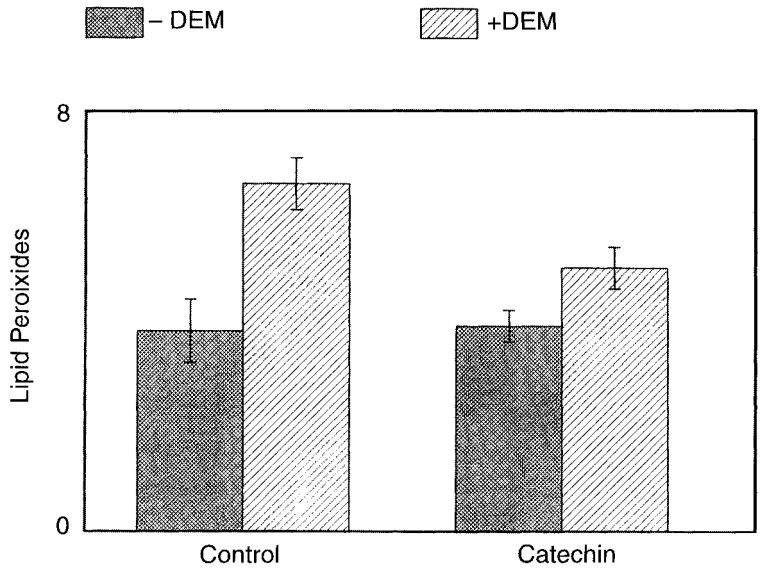

FIGURE 8.2 Catechin and diethylmaleate (DEM)-induced lipid peroxidation. Rats drank water ± catechin (2.1 g/l) for 45 days. DEM (0.3 ml/kg) in corn oil was administered by intraperitoneal injection twice, 24 h apart with the rats killed 30 h after initial injection. Values are arbitrary units.

do not block skin glutathione depletion caused by sulfur mustard dermal intoxication, but still block lipid peroxidation.[88]

There have been sporadic examinations of flavonoid effects on the antioxidant enzymes glutathione peroxidase and glutathione reductase. Both activities are increased in mouse skin by feeding the isoflavone genistein.[85] The reductase enzyme activity is also increased in the intestine. Another paper[89] reports increases in both enzyme activities in livers of rats fed isoflavone-containing soy protein isolate. In other work, consumption of a green tea extract by mice produces moderately high activities for liver glutathione reductase and very high activities for glutathione peroxidase in three tissues.[90] In humans, plasma glutathione peroxidase activities increase after 10-day consumption of quecetin-rich juices.[38] However, it should be recognized that for this study, as well as those with green tea or soy protein isolates, the effects may not be dependent just on the flavonoids, but may also involve other food components. On the other hand, consumption of pure quercetin by rats does increase gastric mucosal activities of glutathione peroxidase.[83] In addition, in mice, acute treatment with a two-flavonoid combination prevents cardiac glutathione peroxidase activity depletion by the chemotherapy drug adriamycin.[91] This study and the others noted in this paragraph indicate that flavonoids can influence glutathione peroxidase and reductase activities. Nonetheless, there is still a large information gap regarding what flavonoid treatments affect what body sites to what extent. This is especially true from a human study perspective.

There are a few studies of flavonoids and catalase, another antioxidant enzyme. Some of these report that flavonoid feeding does not affect rat liver catalase activities.[92] In contrast, one report states that topical application of silymarin inhibits a depression in skin catalase activity in a mouse photocarcinogenesis model.[93] In addition, two mouse feeding studies mentioned above,[85,90] one with genistein and one with green tea, found increased catalase activities in multiple tissues.

The whole area of flavonoids affecting endogenous antioxidants is interesting, but still requires more research to elucidate the full biological importance of a flavonoid–endogenous antioxidant relationship.

VI. PROOXIDANT EFFECTS OF FLAVONOIDS

Although this chapter has focused on possible antioxidant effects of flavonoids, there are certainly a respectable number of papers concerning prooxidant effects of these same compounds.[94,95] Some of the very chemistry that can make flavonoids free radical scavengers, under certain circumstances, can also generate oxidant reactions. These reactions can damage the same biological molecules that flavonoids are supposed to be able to protect from oxidation. One large issue in this whole area is whether these prooxidant actions can occur *in vivo*, and if so, under what circumstances. So far, the evidence for prooxidant actions of flavonoids has come primarily for studies done *in vitro*. However, if flavonoids continue to gain attention for possible health-promoting effects, the issue of prooxidant actions must also be addressed.

VII. SUMMARY

Many studies give evidence that flavonoid antioxidant actions can impact human health. Yet, this statement still has to be considered a little speculative pending more studies, particularly in humans themselves. There seems to be a number of different mechanisms by which flavonoids can exert direct or indirect antioxidant actions. Exactly which mechanisms operate in which circumstances still requires further illumination. As the antioxidant effects of flavonoids gain more attention, there may also be a need to consider possible prooxidant effects as well.

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9 Carotenoids, Metabolism and Disease

Richard M. Faulks and Susan Southon

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I. INTRODUCTION

Epidemiological studies have consistently indicated that the consumption of fruit and vegetables is inversely related to the incidence of disease, particularly cancer. The correlation also holds true for the carotenoids, but it is unclear if they are just a marker for the consumption of vegetables and fruits, since the majority of carotenoids are derived from these foods.

Mammalian species, including humans, do not have the ability to synthesize any of the carotenoids. Although it is recognized that some bacteria can synthesize carotenoids the microflora of most animals (except ruminants) occurs in the distal part of the digestive tract where carotenoid

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absorption is almost nonexistent. All the carotenoids found in mammalian tissues are therefore derived from dietary sources.

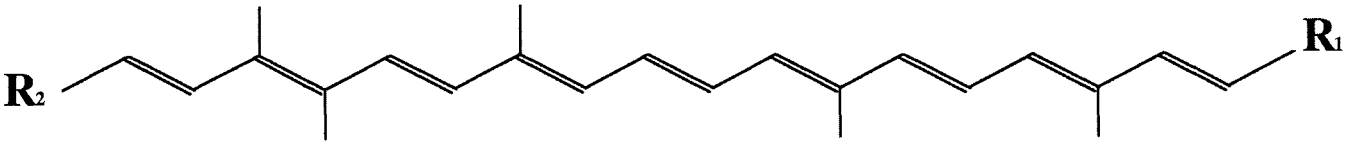
The carotenoids generally found in foods are all-*trans* (E) form C40 polyenes formed from eight 5-carbon isoprenoid units. They may be linear (lycopene), or have undergone ring closure at one end (γ-carotene, citranaxanthin) or at both ends (β-carotene, β-cryptoxanthin, lutein). Where terminal ring structures are present they may carry [OH] or [O] groups giving rise to the hydroxy and keto carotenoids, respectively (Figure 9.1).

Further oxidation of the terminal ring may occur to produce the mono- and diepoxides. The all-*trans* structure is frequently subject to isomerization giving a *cis*- configuration (Z form) at various positions on the polyene backbone. Such configurational change may have a significant effect on the physical and biochemical properties of the molecule. The hydrocarbon carotenoids are apolar lipophylic molecules and show no solubility in water but are readily soluble in organic solvents and to some extent in fats and oils. The presence of an hydroxy or keto group gives the molecule some polarity but such compounds are still predominantly hydrophobic.

In the foods that humans consume, several hundred carotenoids have been identified but there are only a few that are ingested in significant (mg) quantities. These are mainly derived from foods of plant origin, although foods of animal origin, particularly milk, eggs, cheese, liver, kidneys, fat, and processed foods, to which carotenoids have been added for aesthetic or legal purposes, make a contribution.

II. MAIN DIETARY CAROTENOIDS

- **β-Carotene** ($C_{40}H_{56}$, M.wt. 536.9). β,β-carotene. One of the most abundant of the food carotenoids, it is derived from green leaves, where it functions as an photoenergy transfer medium and as a photoprotectant in the light harvesting complex of the chloroplasts. Carrots and red (unrefined) palm oil are major sources. Peaches, apricots, mango, and papaya are the major fruit sources and yellow/orange fleshed varieties of sweet potato (*Ipomoea batatas*), squash (Curcubitaceae), and cassava (*Manihot esculenta*) are major sources in some diets. Most of the world's major cereals have very little β-carotene but small amounts are present in maize (*Zea mays*) and grain legumes. High-carotenoid rice is being developed. Daily intake may vary between 0 and 15mg/day depending on the amount and source of the vegetable foods consumed.
- **α-Carotene** ($C_{40}H_{56}$, M.wt. 536.9). (6′R)-β,ε-carotene. Normally found in the same sources as β-carotene, where it may make up 40% of the total carotenoid content.
- **Lutein** ($C_{40}H_{56}O_2$, M.wt. 568.9). Xanthophyll, (3R,3′S,6′R)-β,ε-carotene-3,3′-diol. The main sources in the human diet are green leafy vegetables. Immature legumes (peas), unripe fruit (green peppers), and egg yolk are also good sources. As its name indicates, it is a dihydroxy carotenoid and the presence of the polar groups alter its properties so that it is easily separated from the hydrocarbon carotenoids. Although present in the free form in leaves, it is normally the acyl (palmitate) esters that occur in fruits and flowers.
- **β-Cryptoxanthin** ($C_{40}H_{56}O$, M.wt. 552.9). (3R)β,β-caroten-3-ol. Citrus fruit and maize are the major sources of this monohydroxy carotenoid in the human diet.
- **Zeaxanthin** ($C_{40}H_{56}O_2$, M.wt. 568.9). (3R,3R') β,β-carotene-3.3′-diol. This dihydroxy carotenoid is mainly derived from maize (*Z. mays*), as its name suggests, although traces are found in many foods. It is chromatographically difficult to separate from its isomer lutein.
- **Lycopene** ($C_{40}H_{56}$, M.wt. 536.9). ψ,ψ-Carotene. Tomato is the main dietary source of this carotenoid, although it is also found in watermelon and pink grapefruit.



Carotenoids	R1	R2
lycopene		
alpha (α) carotene		
beta (β) carotene		
gamma (γ) carotene		

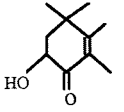
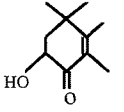
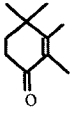
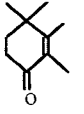
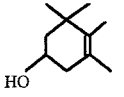
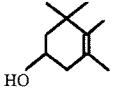
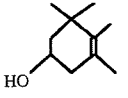
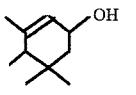
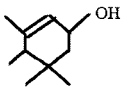
Xanthophylls (Oxycarotenoids)	R1	R2
astaxanthin		
canthaxanthin		
zeaxanthin		
lutein		
cryptoxanthin		

FIGURE 9.1 Structures of the common carotenoids.

Other foods, e.g., *salmo* ssp., Crustaceae, contain astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-dione) and canthaxanthin (β,β-carotene-4,4′-dione) as well as minor amounts of other carotenoids and the ripe red fruit of *Capsicum* spp. contain capsanthin (3R,3′S,5′R)3,3′-dihydroxy-β,κ-caroten-6′-one.

Although, commonly, a particular fruit or vegetable may contain a dominant amount of a specific carotenoid, selection of cultivars can greatly influence both the total amount and relative concentrations of the carotenoids. For this reason, intake data from nutritional studies should be treated with caution and, where possible, backed up by analysis of the dominant carotenoid-containing foods instead of relying on tables of food composition. It should also be noted that in all food plants where carotenoids occur there will also be some of the metabolic intermediates, e.g., phytoene (15-*cis*-7,8,11,12,7′,8′,11′,12′- octahydro-ψ,ψ-carotene), and small amounts of the minor carotenoids.

The conjugated double bonds of the carotenoid molecule, while essential to its function in the plant, make it susceptible to oxidative degradation. In the main, the carotenoid within the food structure is relatively stable but once the structure is disrupted and the carotenoid exposed to heat, light, oxygen, peroxides, transition metals, lipoxygenases, it is rapidly degraded leading to loss of color (bleaching) and biological function.

III. CAROTENOID ABSORPTION

Because the carotenoids are hydrophobic, they are not soluble in the aqueous environment of the gastrointestinal tract. They need to be dissolved/carried in lipid and lipid + bile salt systems to be absorbed at the enterocyte brush border. This mass transfer from the bulk aqueous food to lipid structures is a complex process that is hindered by the presence of food structure but also depends upon whether the carotenoids are present in chloroplasts as lipoprotein complexes, as in the light harvesting complex in plant leaves, or whether it is crystalline in chromoplasts, as in the case of carrot root and tomato fruit. Absorption seems to be assisted by the presence of dietary lipid and digestive enzymes, particularly the presence of lipases. Lipases produce free fatty acids which are incorporated into the mixed micelles (bile salts, lecthin, acyl glycerols, free fatty acids, and minor lipophylic components). Processing carotenoid-containing foods in the presence of fats improves the availability of the carotenoid for absorption, partly because the carotenoids have the opportunity to transfer to the lipid phase prior to ingestion.

Carotenoids are passively absorbed into the enterocyte from the micellar phase of the digesta. However, it is not known if all the carotenoid present in a mixed micelle is absorbed, or whether some (perhaps selectively) is left behind in association with unabsorbed bile salts and cholesterol and is then excreted. Competition between carotenoids for absorption has been noted, with β-carotene suppressing the absorption of lutein. However, more recent studies of absorption of β-carotene and lutein from cooked spinach do not show this effect.

Factors that increase the thickness of the unstirred layer on the surface of the gut, for example, soluble dietary fiber, act as a barrier to the absorption of dietary fats and may, therefore, also limit the amount, or reduce the rate, of absorption of carotenoids. Disease states that impair lipid absorption, for example, cystic fibrosis, celiac disease, vitamin A deficiency, and gut parasites also lead to impaired carotenoid absorption and result in low plasma carotenoid levels, although in some cases persistent inflammation may be a significant factor in reducing plasma levels of carotenoids. The advent of the food use of sucrose polyesters (fat substitutes) like Olestra™ can also lead to a reduction in the absorption of carotenoids.

Studies with Olestra at levels likely to be consumed have been shown to reduce the plasma concentration of β-carotene (20 to 34%), lycopene (38 to 52%), and vitamin E.

Estimates of the absorption of the carotenoids from foods and as isolated compounds vary from a few percent up to 95% depending on the study design, the model used, and the assumptions made in calculating absorption (Table 9.2).

TABLE 9.1
Absorption Table

Carotenoid	%Absorption	Source	Reference
β-Carotene[a]	40–98	Isolate in oil	Bauernfeind and Klaui[2]
β-Carotene[a]	1–87	Raw carrot	Bauernfeind and Klaui[2]
β-Carotene[a]	4–22	Raw carrot, grated	Bauernfeind and Klaui[2]
β-Carotene[a]	1–48	Cooked carrot	Bauernfeind and Klaui[2]
β-Carotene[a]	25–56	Carrot puree	Bauernfeind and Klaui[2]
β-Carotene[a]	9–45	Raw spinach	Bauernfeind and Klaui[2]
β-Carotene[a]	6–88	Cooked spinach	Bauernfeind and Klaui[2]
β-Carotene[b]	75–98	Isolate in milk shake	Faulks et al. 1997[17]
β-Carotene[c]	17–52	Isolate	Shiau et al. 1994[15]
β-Carotene[d]	11–17*	Beadlets	van Vleit et al. 1995[12]
β-Carotene[d]	3.4	Isolate, capsule	O'Neill and Thurnham 1998[11]
Lutein[d]	2.7	Isolate, capsule	O'Neill and Thurnham 1998[11]
Lycopene[d]	2.6	Isolate, capsule	O'Neill and Thurnham 1998[11]
β-Carotene[e]	22	Deuterated isolate	Novotney et al. 1995[13]
β-Carotene[f]	9–17	Isolate, radiolabeled	Blomstrand and Werner 1967[14]

[a] Fecal mass balance
[b] Mass balance in ileostomy volunteers
[c] Gastrointestinal lavage mass balance
[d] Calculated from plasma triacylglycerol-rich fraction carotenoid excursion and hypothetical clearance kinetics
[e] Compartmental model based on plasma concentration excursion
[f] Based on recovery of radiolabel from thoracic duct.
* Assuming central (11%) or eccentric cleavage (17%).

IV. APPROACHES TO MEASUREMENT OF ABSORPTION

A. Metabolic Balance Techniques

The chronic dosing method is dependent on establishing an equilibrium between intake and excretion, and requires fecal markers at the beginning and end of the balance period which is 5 to 8 days. Both intake and excretion values need to be determined with great accuracy because the calculated amount absorbed can be easily confounded by small errors. The use of food tables is insufficient and foods and or supplements need to be assayed accurately (see notes above).

If the method is applied to a single acute dose, the diet needs to be carotenoid-free for 5 days before and during the test period, and fecal collections need to be continued until no further carotenoid from the test dose is lost in the feces. The collection period is usually 3 to 5 days giving a total study period of 6 to 10 days during which there will almost certainly be perturbation of the "normal" plasma concentration of carotenoids due to the modification of the diet.

If these methods (acute or chronic) are used for the measurement of absorption of carotenoids, then it must be assumed that feces are the only significant excretory mechanism of nonabsorbable carotenoids, that there is no enterohepatic recycling, that the carotenoids recovered from the feces are of dietary origin, and that none of the unabsorbed carotenoid has undergone (bio)transformation, or otherwise been lost.

The latter assumption gives cause for concern. The carotenoids are likely to be susceptible both to microbial degradation in the large bowel and to oxidative degradation. Thus, it is likely that unabsorbed carotenoids are not quantitatively recovered from the feces. Much of the carotenoid absorption data from foods and isolates are based on either acute or chronic fecal mass balance methods and show great variability.

B. Ileostomy Mass Balance

In individuals who have undergone ileostomy, the colon has been surgically removed and the terminal ileum brought to a stoma on the abdominal wall. Ingested food passes through the stomach and ileum in around 6 h as it would in the intact individual. The digesta (ileal effluent) can be recovered at regular intervals (2 h) and all the residue from a test breakfast can be recovered in 12 h if the volunteers are given carotenoid-free midday and evening meals. Test meals of either an isolated carotenoid or food can, therefore, be given to an overnight fasted volunteer at breakfast (without dietary modification) and the unabsorbed carotenoid recovered from the ileal effluent in real time without the delay of the colon and rectum, or the confounding influence of the colonic microflora. The model has the added advantage that an excretion profile can be obtained, the timing of which gives a time span for the absorption, which can in turn be compared to changes in plasma concentration over the test period. Using this approach, absorption of isolated crystalline β-carotene given dispersed in a milk shake containing a known amount of fat (10 g) was found to be around 90%, while absorption from a cooked green leafy vegetable (spinach) is around 20 to 30%.

C. Gastrointestinal Lavage Technique

In this technique the entire gastrointestinal tract is washed out (total gastrointestinal washout method, TGWM) by consuming a large volume (1 gal/4.5 l) of "Colyte" containing polyethylene glycol (PEG) and electrolyte salts. Washout is complete with the production of clear rectal effluent (2.5 to 3.5 h). The volunteers then consume the test meal and are permitted only water or "diet" soft drinks (noncaloric) for the next 24 h. All the effluent is collected and pooled with the effluent collected on the following day when another dose of Colyte is given to wash out the remainder of the test meal. The carotenoid recovered in the stool is subtracted from that fed to obtain an absorption figure. Absorption values for isolated β-carotene of 17% were obtained in the absence of a meal and 52 to 29% with a meal. Difficulties associated with the method is that it is relatively time-consuming, can only be applied to healthy individuals, and may give an underestimation of absorption if absorption is compromised or normal transit time is reduced due to the use of "Colyte." In addition, as with the fecal mass balance, the method depends upon there being no degradation or loss of unabsorbed carotenoids. On the other hand, it has the advantage of standardizing the residence time of carotenoids in the gastrointestinal tract.

D. Plasma and Plasma Fraction Concentration Methods

Measurements of absorption are usually carried out by the administration of an acute or chronic dose of isolated carotenoid, or carotenoid-containing food, and following the changes in plasma concentration of the carotenoid of interest.

1. Acute Doses

Measurements are usually carried out in fasted individuals who have restricted their dietary intake of the carotenoid of interest (and other carotenoids) for several days before the test day and for a period of days following. This method cannot determine absolute absorption but it is possible to compare different doses and foods and derive some information regarding the relative absorption by comparison with a standard dose, normally the isolated carotenoid. Such studies cannot normally be carried out blind because of the problem of disguising the treatment. A crossover design, with an adequate period of washout between treatments, is the most suitable approach so that each individual can act as his or her own control (paired t-test).

The measurement of absolute absorption of a carotenoid, calculated from the changes in plasma concentration following a single acute dose, is difficult and frequently misunderstood. The first point to deal with is the form and duration of the plasma response curve. Peak plasma concentration

occurs between 6 and 48 h, depending upon the dose and the frequency of measurements. Since it is evident that the dose passes through the ileum in about 6 h, the advent of plasma peaks found beyond this time can only result from delayed passage of carotenoid to the serosal side after absorption into the enterocyte, or rapid absorption of carotenoid into the body, sequestration from the circulation, and then reexportation to the plasma.

Evidence cited for the first case is a frequently found second plasma peak occurring following a meal. However, this is countered by lack of evidence for temporary storage in the enterocyte. There is no known storage mechanism, no "tailing" of ileal loss in ileostomy patients and radiolabeled β-carotene absorption appears complete in less than 12 h. The second peak could simply result from an increase in the plasma lipids following a meal, providing the lipoprotein and triglyceride needed to transport the carotenoid in the plasma. Alternatively, and most probably, the first peak in plasma concentration is due to the carotenoid present in the newly absorbed chylomicrons and the second peak, or prolonged duration of the first peak, results from hepatically reexported carotenoid in very low-desity lipoproteins (VLDL) and low-density lipoproteins (LDL). Plasma sampling times should be carefully chosen to ensure that a true reflection of changes in concentration are acquired.

The transfer of carotenoids from the short-lived chylomicrons to the longer-lived LDL and HDL (which appear to carry most of the carotenoid in fasting subjects) would also explain why the plasma concentration may remain elevated for up to and beyond 10 days after a dose. Under such circumstances, the use of the *A*rea *U*nder the plasma *C*urve (AUC) approach is not appropriate for the calculation of absolute absorption because the kinetics of absorption, disposal, and reexport are not known.

2. Chronic Dosing

Chronic dosing with supplements or foods needs to be carried out until the plasma concentration reaches a plateau. This normally takes a period of weeks when supplementing with dietary achievable amounts (15 mg/day) and may increase the plasma concentration of β-carotene up to tenfold, with other common carotenoids showing smaller increases. It should be noted that the size of the increase in plasma concentration is not necessarily related to differences in absorption since change in plasma concentration is the difference between absorption and clearance. Again, absolute absorption cannot be measured, but the data may allow comparisons between isolated compounds and foods, and between different foods. It should be noted that such comparisons assume that there is a known (linear) relationship between the dose and the plasma excursion. This may generally be true for small doses, but larger doses (>15 mg) may exceed the capacity of the body's normal handling mechanisms, leading to changes in the relative ratios of pool sizes and thus leading to nonlinear responses.

Decay curves of falling plasma concentration of carotenoids, when supplementation is discontinued, may also provide some useful data on the half-life of the body carotenoid pool and can be used to calculate body pool half-life and clearance rates.

The comparison of the absorption of one carotenoid with another is not possible unless the absorption and disposal kinetics are known.

E. Plasma Triglyceride Rich Lipoprotein Fraction

Newly absorbed carotenoids are initially present in plasma chylomicrons before they are sequestered by body tissues and reexported in, or transferred to, other lipoprotein fractions (Figure 9.2). Thus, measurement of carotenoids in this fraction, and a knowledge of the rate of clearance from the chylomicrons, should permit the calculation of rates of absorption, disposal, and overall absorption based on AUC measurement. This method has the advantage that chylomicrons present in fasting plasma are few and they are almost devoid of carotenoids. The disadvantage is that the plasma has

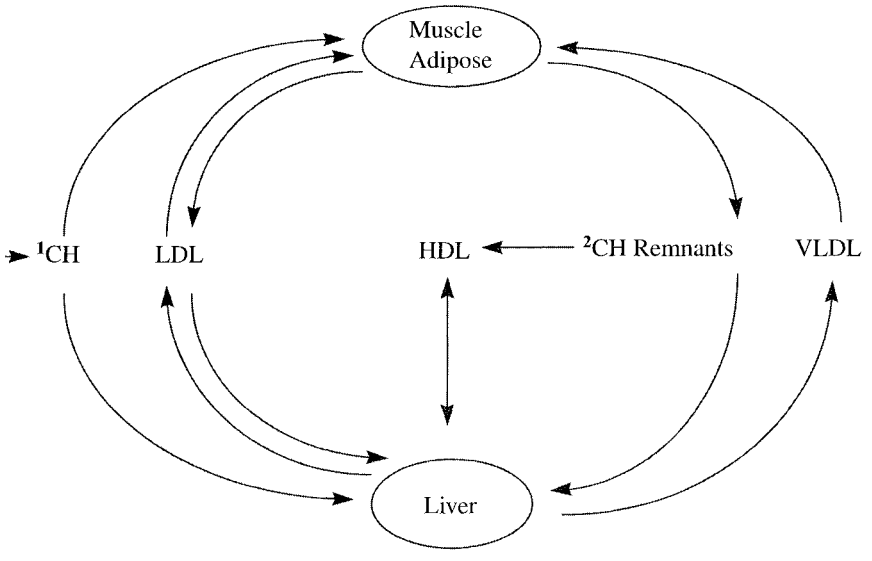


FIGURE 9.2 Bioavailability and kinetics of carotenoids in lipoprotein carriers. $^1 t_{1/2} = 2$ to 5 min; $^2 t_{1/2} = 11.5$ min.

to be ultracentrifuged to separate the lipoprotein classes. Ultracentrifugation, however, does not normally permit the separation of the chylomicron fraction free of other LDLs, particularly the VLDL, which may be the primary vehicle for the hepatic reexport of absorbed carotenoids.

In addition, oral absorption data based on triglyceride-rich lipoprotein (TRL) area under the curve, and the theoretical AUC that would be obtained if the dose had been administered intravenously (using plasma volume and chylomicron clearance half-life), give results that differ. For β-carotene, an absorption figure of 11% (central cleavage) or 17% (eccentric cleavage) has been reported, whereas other researchers calculated 3.9 and 2.5% absorption in males and females, respectively, assuming only central cleavage. Both authors assume a cited chylomicron remnant half-life of 11.5 min. However, a true clearance rate of carotenoid in the TRL fraction can also be obtained from the graph of TRL carotenoid concentration against time beyond the absorption phase, and this could be used to provide a true carotenoid half-life term which would be independent of assumptions based on lipid kinetics.

It is also worth noting that much shorter half-lives (2.5 to 7.9 min) have been reported for the clearance of chylomicron triglyceride and use of these values, rather than those of chylomicron remnant clearance, would have the effect of proportionally increasing the apparent absorption (% absorption doubles every time the half-life is halved).

The calculation of absorption, using a theoretical plasma concentration excursion based on plasma pool size and a theoretical intravenous dose, must be treated with caution unless exact clearance kinetics of the carotenoid are known.

Some difficulties in explaining carotenoid kinetics may arise from the observations that the triglyceride response in the TRL peaks at around 2 h, whereas the β-carotene peaks at 5 to 6 h. Individuals are highly variable in their plasma and TRL response to oral β-carotene and individuals with a high concentration of plasma β-carotene appear to be those who show the greatest increment in plasma carotenoid concentration on supplementation. The use of TRL is, currently, a useful option for the calculation of β-carotene bioavailability, but its use for the other carotenoids has yet to be tested.

Carotenoid–carotenoid interactions and the observation that the carotenoid profile in the TRL fraction is not the same as in a supplement clearly indicate that to assess bioavailability of any one carotenoid the carotenoid profile of the supplement or food needs to be defined, as do the amount and type of fat in the test meal. The supplementation (both acute and chronic) of volunteers with β-carotene has been found to suppress plasma lutein and the ratio of *cis*:*trans* isomers of lycopene

and β-carotene in the plasma seem to be consistent irrespective of the ratio in the supplement. It has been shown that isomerization of all *trans* β-carotene to 9-*cis*-β-carotene can occur in the human mucosa, so perhaps there is a regulatory mechanism which controls the relative ratios of *cis*:*trans* isomers. This means that if a single isomer is fed and several isomers appear in the plasma, there is no way of knowing how much has been isomerized other than by the use of tracers. In such a case, plasma measurement of a single isomer as given orally will be inadequate for absorption measurements.

F. Isotope Methods

The use of radioactive tracers in human volunteers, to determine the bioavailability of the carotenoids, is not now possible because of ethical constraints. There are, however, two studies in men using ^{14}C and ^{3}H. These studies provide useful information on the duration and extent of absorption of β-carotene and the degree of conversion to retinol. Absorption of radiolabeled β-carotene was found to be in the range 8.7 to 16.8%, but most of this was recovered as the retinyl esters (see comments on retinol equivalents). This indicated that β-carotene absorbed by this route was largely converted to retinol. Peak absorption was found to be at 3 to 4 h and of 6 to 7 h for each of two volunteers, respectively, and this time coincided with maximum lactescence in the lymph as assessed visually (see comments on triglyceride response). In both cases, despite the relatively low absorption, no further radiolabel was found in the lymph after 12 h. Transitory storage in the enterocytes, prior to transfer to the serosal side, would probably have been detected as a tailing of the absorption curve and the high level of conversion may partly explain why elevation of plasma β-carotene is not always seen in volunteers given small acute doses.

The use of stable isotopes is more ethically acceptable. Highly labeled β-[^{13}C] carotene has been used to study the metabolism of β-carotene in humans. The single acute oral dose used in these studies was 1 to 2 mg of purified labeled (>95% ^{13}C) carotene, dissolved in tricaprylin or safflower oil and given with a standard meal. Blood samples were drawn at intervals and the β-carotene, retinol and retinyl esters separated, quantified, and purified by high-performance liquid chromatography (HPLC). The β-carotene (converted to the perhydro derivative by hydrogenation over platinum oxide), and the retinol and retinol-derived from retinol esters, were subjected to gas chromatography-combustion-isotope ratio mass spectrometry (GCC-IRMS). The method was sufficiently sensitive to track the ^{13}C in retinol esters up to 2 days and β-carotene and retinol up to 25 days.

By making some assumptions regarding the rate of clearance of β-[^{13}C] carotene, and the retinol derived from it, it was estimated that about 64% of the ^{13}C entered the plasma as retinyl esters, 21% as retinol, and 14% as β-[^{13}C] carotene. The very high level of conversion of the β-[^{13}C] carotene is in line with the data from the radiolabeled β-carotene studies described above. It may be that small doses of β-carotene used in these studies show these high proportions of conversion to retinol but if this percentage conversion were maintained in supplementation studies toxic levels of retinol could result.

Potentially, the use of β-[^{13}C] carotene, either as an isolate or within a food, should permit the measurement of absolute absorption and the kinetics of disposal and conversion to other metabolites. An alternative to the use of β-[^{13}C] carotene is octadeuterated β-carotene (β-carotene-d_8), an isotopomer that can be separated from natural abundance β-carotene by HPLC, thus avoiding the use of mass spectrometry. The retinol-d_4 derived from β-carotene-d_8 has to be separated from the plasma using a solid phase system and derivatized to the tert-butyldimethylsilyl ether before measurement by CG-MS. The method has been applied successfully to the tracking of both β-carotene-d_8 and retinol-d_4 in human volunteers for up to 24 days after an oral dose of 73 μ*M* (40 mg). Application of a compartmental model indicated that 22% of the carotenoid dose was absorbed, 17.8% as carotenoid and 4.2% as retinoid. This result is close to the 11% absorption of β-carotene found previously but indicates a much lower percentage conversion to retinol than that found using very small oral doses of β-[^{13}C] carotene.

V. DISTRIBUTION AND METABOLISM OF CAROTENOIDS

Once the carotenoid is inside the enterocyte, it is transported to the serosal side, packaged with triglyceride and protein into chylomicrons, excreted into the mesenteric lymphatics, and enters the subclavian veins via the thoracic duct. There have been suggestions that some of the xanthophylls may be carried in the portal blood but as yet there is no evidence for this. Unlike most water-soluble nutrients, which are transported to the liver in the portal blood, the chylomicrons enter the general blood circulation and are acted on by the endothelial lipoprotein lipases in the extrahepatic capillary bed. The lipases hydrolyze the triglyceride in the chylomicron to free fatty acids which are absorbed by the immediate tissues. The chylomicron remnants and glycerol are cleared by the liver. It is unclear whether the carotenoids are sequestered from the circulation in the extrahepatic capillary bed or are cleared from the plasma with the chylomicron remnants.

Absorption studies using AUC for carotenoid concentration in chylomicrons generally use the accepted value for the half-life of chylomicron remnants ($t_{1/2}$ =11.5 min) although there is evidence that the clearance kinetics of β-carotene are more rapid ($t_{1/2}$ = 2 to 5 min), implying that at least some of the carotenoid is absorbed in the extrahepatic capillary bed along with the fatty acids. A study of the relative ability of the chylomicron and the chylomicron remnant to carry the carotenoid would help to resolve this point.

A. Distribution

Once absorbed, the carotenoids are reexported by the liver in VLDLs, which are subsequently metabolized to low-, intermediate-, and high-density lipoproteins (LDL, IDL, HDL). The lipoprotein classes carry different profiles of carotenoids. Lutein is distributed about 60:40 while β-carotene and lycopene are distributed 20:80 in HDL and LDL, respectively, in fasting plasma. It is not known if this distribution is biologically controlled, or is simply a thermodynamically favored distribution, as there is no known carrier/binding protein in the plasma. It has been suggested that the apolar carotenoids are carried in the hydrophobic core of the lipoproteins but that the polar carotenoids are more likely present at the surface. This physical difference may allow the exchange of the polar carotenoids between lipoprotein classes.

The HDL has a much longer half-life than LDL so it would be expected that the turnover of the carotenoids preferentially carried by HDL (lutein) would be slower. The carotenoids are variously distributed in the body with body fat containing about 80% and liver 10% of the total body load. The retina of the eye contains high levels of the xanthophylls, lutein, and zeaxanthin in association with binding proteins.

B. Toxicity

As far as can be ascertained, the carotenoids are not cyto- or genotoxic in either large acute doses or in chronic supplementation studies lasting several years. Carotenoderma (yellowing of the skin) is commonly encountered in supplementation studies and in individuals who consume large quantities of carrot or carrot products, particularly if they have a low body mass index (BMI). The condition is rapidly reversible after ceasing intake. Conversion to retinol is controlled so that vitamin A toxicity does not occur. However, consumption of high levels of lutein, zeaxanthin, and cantaxanthin, which are accumulated in the eye, may lead to problems.

C. Metabolism

In human serum, approximately 34 different carotenoids have been identified. Some of these carotenoids are found in the foods consumed, while others appear to be *cis*-isomers or oxidation products produced *in vivo*. The main carotenoids generally found are β-carotene, 13- or 9-*cis*-β-carotene, lutein, zeaxanthin, β-cryptoxanthin, lycopene, and 5-*cis*-lycopene. Once absorbed into

the enterocyte, the carotenoids, which may act as retinol precursors, may be cleaved. β-Carotene, the main precursor of vitamin A, theoretically produces two molecules of retinal (central cleavage), which is reduced to retinol or oxidized (irreversibly) to retinoic acid, while eccentric cleavage only gives rise to apocarotenals in which the conjugated polyene is successively shortened by β-oxidation to one molecule of retinoic acid. Such theoretical yields are not normally observed and the average retinol equivalent of β-carotene is taken as 6:1 (wt:wt) or 3:1 as a molar ratio. It is unclear what the relative contributions of absorption and conversion make to this figure, but recent studies with small doses of stable isotope (^{13}C)-labeled β-carotene give a conversion ratio of 2.3:1 (wt:wt). This would indicate an absorption efficiency of around 40%. Conversion to retinol is dependent on the presence of a β-ionone ring and all carotenoids possessing such a structure have potential vitamin A activity. Minor carotenoids with only one β-ionone ring (α-carotene) are assigned a retinol equivalent of 12:1 (wt:wt). Retinol produced in the enterocyte is esterified before being exported in the chylomicrons and then into the blood from which it is sequestered and stored by the liver. During this process, there is generally no change in plasma retinol.

It should, however, be noted that the plasma concentration of retinol is strictly controlled to the extent that carotenoderma may occur with chronic high β-carotene intake without significantly altering the plasma retinol concentration. Conversely, in retinol-deficient individuals, both the absorption and conversion of β-carotene to retinol may be much higher (see comments on conversion to retinol). The use of the 1/6 conversion factor for retinol equivalents from β-carotene should, therefore, be treated only as a very rough guide.

The 9-*cis*-β-carotene gives rise to 9-*cis*-retinoic acid which has retinoid activity and 11-*cis*-β-carotene yields 11-*cis*-retinol which is central to the regeneration of rhodopsin in the eye. It is not known if other β-carotene *cis*-isomers produce biologically active retinoids. There is currently some debate whether or not some of the other carotenoids can give rise to other biologically active retinoid analogues.

β-Carotene and other carotenoids with retinol potential that are not cleaved in the enterocyte may be cleaved in the liver, kidney, and possibly other tissues that have the necessary enzyme, 15,15′-β-carotenoid dioxygenase (EC 1.13.11.21), and there is some debate regarding the relative contribution of the different possible sites of cleavage.

D. Antioxidant Reaction of Carotenoids

- *Singlet energy transfer:* Carotenoids in the chloroplast act as antenna pigments by their ability to become singlet excited by a much broader spectrum of light than chlorophyll. The excited carotenoid can then pass on the energy to produce singlet excited chlorophyll which can then undertake to process of photosynthesis.

$$\text{Carotenoid} + \text{light} \rightarrow {}^{1}\text{Carotenoid}$$

$${}^{1}\text{Carotenoid} + \text{Chlorophyll} \rightarrow \text{Carotenoid} + {}^{1}\text{Chlorophyll}$$

- *Triplet energy transfer:* Under some conditions molecules can absorb light to produce triplet excited states. The carotenoids, because of their polyene structure, are able to absorb the excitation energy to become triplet excited and then decay exothermically to their ground state, thus preventing the production of potentially damaging radicals.

$$\text{Molecule} + \text{light} \rightarrow {}^{3}\text{Molecule}$$

$${}^{3}\text{Molecule} + \text{Carotenoid} \rightarrow {}^{3}\text{Carotenoid} + \text{Molecule}$$

$${}^{3}\text{Carotenoid} \rightarrow \text{Carotenoid} + \text{Heat}$$

- *Singlet [O] quenching:* Light or chemical action can convert ground-state oxygen ($^3O_2$) to singlet oxygen ($^1O_2$) which is extremely reactive. Singlet oxygen can be quenched by reacting with the carotenoid to produce triplet-excited carotenoid which decays exothermically as before.

$$^1O_2 + \text{Carotenoid} \rightarrow {}^3O_2 + {}^3\text{Carotenoid}$$

$$^3\text{Carotenoid} \rightarrow \text{Carotenoid} + \text{Heat}$$

E. Reactions with Radical Species

The conjugated polyene backbone of the carotenoids has the ability to delocalize a charge or an unpaired electron. These physical chemical properties confer the ability to act as an antioxidant and to terminate free radical reactions *in vitro* with the production of resonance-stabilized free radical structures.

Termination may be as a result of (1) adduct formation, where the free radical joins onto the polyene chain to produce a much less reactive free radical, (2) electron transfer from the carotenoid to the free radical to produce a less reactive charged carotenoid radical, or (3) donation of a hydrogen molecule to the free radical to produce a stable carotenoid radical.

F. Erythropoitic Protoporphyria

Both β-carotene and cantaxanthin have been used to reduce photosensitivity in erythropoietic protoporphyria, although there is a risk of cantaxanthin crystals forming in the eye (cantaxanthin retinopathy).

G. Age Related Macular Degeneration

The eye is the only human tissue where it has been demonstrated that there are specific proteins that can bind carotenoids. Age-related macular degeneration (AMD) is one of the leading causes of age-related irreversible blindness in otherwise healthy individuals. The retina of the eye contains two xanthophylls, lutein and zeaxanthin in equal proportions although the zeaxanthin, is found mainly in the macular region and the lutein throughout the retina. Their function in the eye appears to be as photoprotectants since they can quench singlet oxygen and inactivate triplet-excited molecules (see above) produced by light, thus reducing the oxidative stress on the eye proteins.

Epidemiological studies of AMD and intake of the xanthophylls have not confirmed an association; however, it has been shown that older subjects with low densities of macular pigment have impaired visual sensitivity, whereas those with normal pigmentation have similar visual sensitivity to younger subjects. There is some evidence that supplementation with lutein can increase the density of macular pigment but the impact on AMD is not known.

VI. CARDIOVASCULAR DISEASE AND CANCER

Much has been made of the antioxidant properties and it has been postulated that *in vivo* they can (1) break free radical chain reactions that may oxidize unsaturated lipids and (2) protect DNA from free radical attack. These two processes are seen to be central to the initiation and progression of atherogensis (atherosclerosis) and cancer, respectively.

A. Atherogenesis

The postulated mechanism for the development and progression of this disease is the production of oxidized LDL because of impaired antioxidant status. The oxidized LDL is taken up by monocytes

which infiltrate the artery wall and differentiate into macrophage which vigorously scavenge oxidized LDL to produce foam cells. The foam cells cause the initial fatty streaks in the arterial wall which build up to form the plaque characteristic of vascular disease.

Circulating antioxidants and antioxidants within the LDL are believed to help prevent the LDL and associated apoprotein B from being oxidized by fatty acid hydroperoxides.

The carotenoids are implicated in this process because they are believed to be capable of preventing the formation of lipid hydroperoxides by breaking the free radical propagation of lipid oxidation and because they are contained within the LDL particle itself and can therefore act "on site." Whether or not the carotenoids can act to prevent the early stages of lipid peroxidation and provide antioxidant protection to LDL is actively being researched, but as yet has not provided any convincing evidence for this mechanism.

B. Cancer

Perhaps the most widely researched issue is the relationship between the carotenoids and the induction and progression of cancers. In some models the carotenoids have been shown to have beneficial effects with regard to cancer initiation, progression, and proliferation at some sites. The mechanism of action may be upregulation of cell–cell communication, apoptosis induced by retinoic acid or other metabolites, protection against initial transformation by carcinogens by protecting DNA from free radical attack, and upregulation of immune function.

The carotenoids, including the non-provitamin A carotenoids, have been shown to upregulate the expression of the connexin 43 gene in cell culture leading to greater cell–cell communication. It has been proposed that this has the effect of suppressing cells that have undergone transformation because they are surrounded by, and in communication with, normal cells. These aberrant cells are therefore, at least initially, prevented from progressing to overt cancer. The carotenoids have the ability to suppress the proliferation of cells with chemically induced neoplastic changes in that if the carotenoid is subsequently removed neoplastic foci develop. No such protection is seen in carotenoid-treated cells subjected to irradiation or in cells treated with carotenoid after carcinogen-induced production of neoplastic foci. It seems therefore that the carotenoids can reversibly suppress the development of chemically but not radiation induced neoplastic foci. Upregulated cell–cell communication may be a mechanism by which oral doses of β-carotene may reverse leucoplakia, a premalignant lesion of the oral cavity, although β-carotene supplementation may also increase plasma levels of tumor necrosis factor alpha (TNF-α). A prerequisite of cell–cell communication via gap junctions is that the cells are juxtaposed and cannot move with respect to each other. It would not be surprising therefore to find that the carotenoids/retinoids are also involved in intercellular adhesion, tight junctions, and cell–cell recognition.

The retinoids are particularly potent agents in cell differentiation and there is a considerable body of evidence that they are able to promote programmed cell death (apoptosis) of transformed cells both in cell culture and *in vivo*, but as yet there is no evidence that apoptosis can be initiated by the presence of the non-provitamin A carotenoids.

As has already been pointed out, the carotenoids are effective agents in the scavenging of free radicals. This attribute is postulated to protect DNA from damage by base corruption (oxidation, deletion, adduct formation). In normal cells, corrupt DNA is repaired by a group of enzymes that can excise the damaged base (causing single strand breaks), insert a new base molecule, and reconnect the strand of DNA (ligation). The increase in DNA strand breaks, as measured by the "Comet" assay, in peroxide-challenged cells (*ex vivo*) from subjects treated with carotenoids can therefore be interpreted as an increase in damage susceptibility or upregulated repair (excision and ligation). This dilemma, coupled with the known pro-oxidant properties of the carotenoids at high oxygen tension, is difficult to resolve but the balance of evidence is probably in favor of upregulated repair, although any genetic mechanisms remain to be elucidated.

VII. OTHER METABOLIC ISSUES REGARDING CAROTENOIDS

There is very little information on the metabolic fate of the non-provitamin A carotenoids or the provitamin A carotenoids that are not metabolized to retinol. It is assumed that they undergo oxidation (photobleaching in the skin?), cleavage, and polyene chain shortening by a process analogous to the β-oxidation of fats and that the unmetabolizable remnants are detoxified in the liver by the addition of sugar residues and are then eliminated in the feces (enterohepatic circulation) and urine. Excretion of carotenoids via enterohepatic circulation has not been observed in ileostomy volunteers in that the ileal effluent is virtually free of carotenoids when volunteers are fed a carotenoid-free diet. The observation that large intakes of carotenoids can result in yellowing of the skin might suggest that the skin is a significant excretory mechanism.

Despite the obvious bioactivity of carotenoids and related compounds in model systems, human intervention studies have not given convincing evidence that the incidence of chronic disease is significantly affected by carotenoid intake per se. It may be that with adults the primary neoplastic and atherosclerotic changes have already occurred or that the course of the disease is not generally amenable to treatment by large-dose, short-term supplementation. Focusing on children and young adults, and the prevention of initiation of chronic disease would be a more fruitful exercise.

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10 Lycopene: Source, Properties and Nutraceutical Potential

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I. INTRODUCTION

Over the past decade, certain plant substances, which have come to be known as phytochemicals, have been the focus of considerable attention because of their potential health benefit. Lycopene is one such substance, which belongs to a broad class of lipophilic compounds referred to as the carotenoids. This interest has been partly stimulated by the growing amount of research pertaining to the health benefits of fruit and vegetable consumption. Early explorations of lycopene stemmed from scientific curiosity of the deep yellow, orange, and red pigments which are produced by the various carotenoids. Lycopene is produced by certain fruit and vegetables, especially during the ripening phase.

Although lycopene was initially explored during the early 1900s, it was not until 1930 that it was realized that certain carotenoid compounds were metabolic precursors of vitamin A.[1] However, this ability to provide provitamin A activity was found to be limited to those carotenoid molecules that contain an unsubstituted β-ionine group. Lycopene is one such carotenoid lacking this β-ionine group and thus lacks provitamin A activity. On the other hand, β-carotene, α-carotene, and β-cryptoxanthin possess provitamin A activity. Approximately 50 years later, epidemiologists reported that an inverse relationship existed between diets rich in red, orange, and yellow fruit and vegetables and the risk for developing various forms of cancers as well as other chronic diseases. At first, it was concluded that these effects were produced from the elevated vitamin A activity in the diet. However, upon closer examination it was revealed that the food tables used to form these conclusions converted the content of provitamin A carotenoids into vitamin A values. These results catalyzed much of the modern research interest of the health benefits of other carotenoids in the diet. Until recently, it was believed that many of the health effects were only attributable to the provitamin A compounds. However, lycopene possesses no provitamin A activity, but has been found to have many beneficial biological effects. The deep red crystalline pigment produced by lycopene was

0-8493-8734-5/01/$0.00+$.50
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first isolated in 1873 by Harten from *Tamus comunis* L. berries.[2] Lycopene belongs to a class of more than 60 carotenoids and over 600 fat-soluble pigments.[3] It is also the most abundant non-provitamin A compound present in the Western diet and human plasma.[4]

II. LYCOPENE — AN OVERVIEW

Lycopene is one of the major carotenoids that can be found in the human serum and various tissues throughout the body. Recent research has shown that lycopene may present itself as a biological agent which may play a role in chronic disease reduction or prevention. There are more than 60 carotenoids found in nature, which can be segregated into two general classes. These classes are comprised of the hydrocarbon carotenes and their oxygenated xanthophyll derivatives, with lycopene belonging to the former. From a molecular standpoint, lycopene is nonpolar and is generally found as an acyclic carotenoid containing only hydrogen and carbon (Figure 10.1).[5] Approximately 95% of the time, its 11 conjugated double bonds can be found linearly arranged in the all-*trans* conformation.[2] It is hypothesized that each of these 11 double bonds can undergo isomerization and potentially produce various mono- or poly-*cis* isomers.[6] These isomers may be created as a result of light absorption, heat exposure, and by participation in certain chemical reactions. The all-*trans* conformation is the predominant species found in tomato and tomato products. Various geometric isomers have also been identified. Isomers that have been identified in the human serum include 15-*cis* lycopene, 13-*cis* lycopene, 7-*cis* lycopene, and 5-*cis* lycopene. In fresh tangerine-type tomatoes, 7,9,9′,7′ lycopene has also been isolated. One last isomer which has been identified is 11-*cis* lycopene. Unlike several of the other carotenoids studied to date, such as α-carotene and β-carotene, lycopene exhibits no provitamin A activity. This is due to the simple fact that lycopene lacks the β-ionone ring structure. Although it possesses no provitamin A activity, it bears exceptional antioxidant activity relative to the other carotenoids.[7]

A. Antioxidant Properties

The acyclic structure of lycopene, its various conjugated double bonds, and its rather high hydrophobicity provided the foundation for speculation that it may exhibit various unique biological characteristics.[6] It has been proposed that the antioxidant capabilities of the carotenoids are the basis for their protective effects against cancer.[8] *In vitro* studies of carotenoids, such as lycopene, have determined that these molecules are effective biological antioxidant agents because of their singlet oxygen quenching ability, their good radical-trapping properties, their effectiveness in scavenging peroxyl radicals, or some combinations of these qualities. Lycopene has been determined to be the most efficient biological carotenoid singlet oxygen quencher.[7] The quenching ability is closely related to the number of conjugated double bonds that this compound contains. It is this fact that makes lycopene, compared with the other C-40 carotenoids, the most effective singlet oxygen quencher because lycopene contains two additional double bonds.[5]

Lycopene and other carotenoids are believed to provide protection against free radical damage.[9] Molecules with unpaired electrons in the outermost orbital are commonly referred to as free radicals. Free radical formation begins with the addition of a single electron to O_2 which in turn produces the superoxide radical (O_2^-). A subsequent addition of an additional electron and two hydrogen ions to superoxide results in the formation of hydrogen peroxide (H_2O_2). One further reaction of

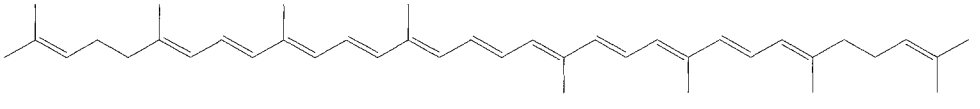

FIGURE 10.1 Lycopene.

hydrogen peroxide with a single electron and an additional hydrogen ion will then generate a free hydroxyl radical (OH•). It is these hydroxyl radicals that pose great concern because of their great magnitude of reactivity with the surrounding environment. They are capable of producing damage to proteins, lipids, and DNA.

Another highly reactive, short-lived oxygen species that is drawing recent attention is singlet oxygen ($^1O_2$). Although not a true free radical by definition, singlet oxygen has the ability to react with various biomolecules.[6] Lycopene is believed to quench singlet oxygen through physical or chemical processes. The physical quenching process is typically more effective and occurs a majority of the time. In this process the carotenoid remains undamaged after a transfer of energy from the singlet oxygen to the carotenoid, thus allowing itself to undergo further cycles of singlet oxygen quenching. This yields the production of a ground-state oxygen and lycopene in the excited triplet state. Alternatively, a bleaching or decomposition of the carotenoid occurs via chemical quenching. It is believed this latter process of singlet oxygen quenching contributes to less than 0.05% of the overall quenching activity.[5] Lycopene has also been reported to provide exceptional protection to lymphocytes from nitrogen dioxide radical cell death and membrane damage.[10,11]

Much work still needs to be conducted regarding the metabolism of lycopene. Few metabolites in the serum and tissues have been documented in the literature. One metabolite, 5,6-dihyroxy-5,6-dihydro-lycopene, has been identified to be the result of the oxidation of lycopene to a lycopene epoxide intermediate.[8] To determine other small lycopene metabolites, it may be necessary that additional efforts be made with analytical techniques to develop new tools that can detect these substances present in the serum and tissues.

B. Diet Sources

In the United States, it is believed that lycopene contributes to approximately 30% of the total carotenoid intake, which equates to about 3.7 mg/day.[12] For comparison, it has been estimated that daily lycopene intake in Great Britain is 1.1 mg.[13] It has also been reported that the mean lycopene intake has increased by 5 to 6% among American adults 18 to 69 years between the years of 1987 and 1992.[14] Analysis of an individuals' 3-week food diaries determined a strong correlation for exogenous lycopene intake and plasma concentrations.[12] However, no correlation was reported for lycopene and fruit and vegetable consumption.[15] These results are suggestive that lycopene may not be a suitable marker for total fruit and vegetable intake and that lycopene may not be well represented in all vegetation.

What makes lycopene exceptionally different from the other carotenoids is that it is primarily represented by a single dietary source: tomatoes and tomato products (Figure 10.2).[4] In the United

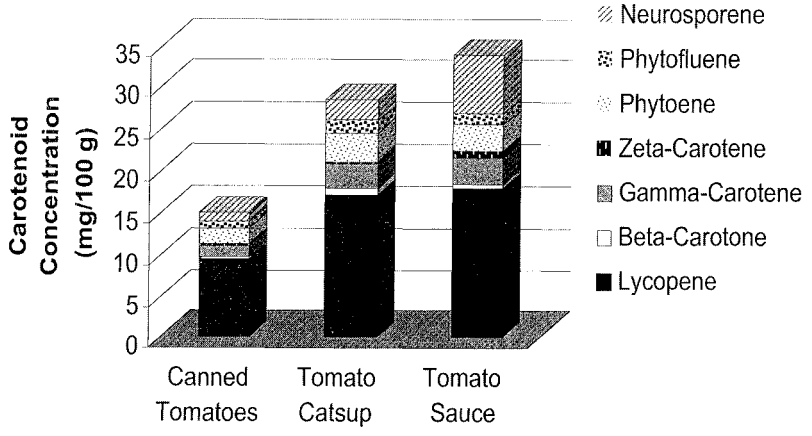

FIGURE 10.2 Various carotenoids found in tomatoes and tomato products. (Data adapted from Reference 4.)

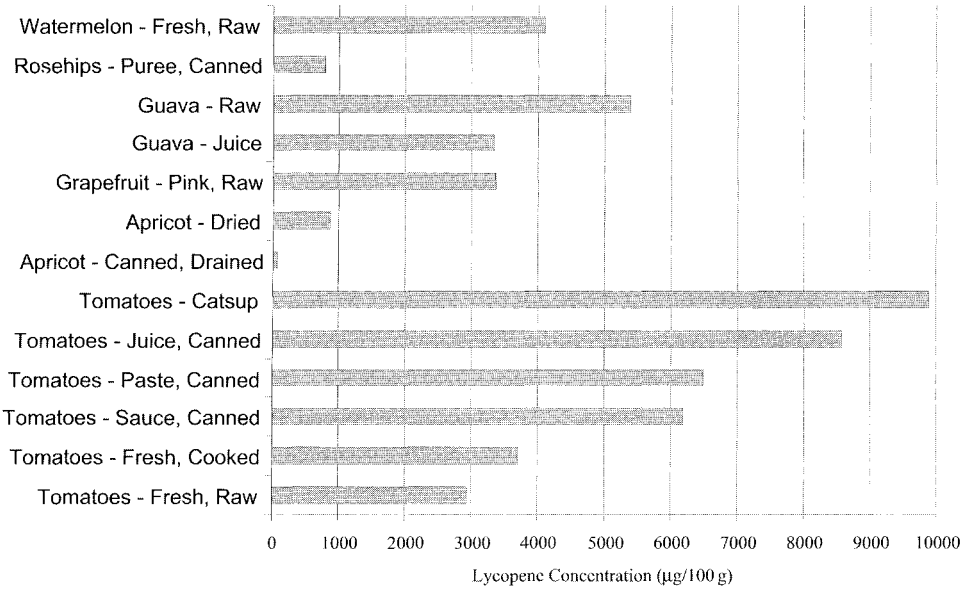

FIGURE 10.3 The concentration of lycopene found in various fruit and vegetables.

States, more than 80% of the total lycopene intake is derived from the ingestion of tomato products.[6] The ripeness of the fruit can cause variations in lycopene concentrations in these foods. Varieties of tomato products that are redder possess a lycopene concentration of approximately 50 mg/kg, whereas yellower varieties have a lycopene content of about 5 mg/kg. Tomatoes have been reported to contain 30 mg of lycopene for each kilogram of raw fruit.[5] It has been reported that lycopene represents a substantial portion of the total carotenoid content of tomato products.[6] It is estimated as much as 60 to 64% of the total carotenoid content consists of lycopene. Even greater quantities of lycopene have been observed in tomato juice (150 mg of lycopene per liter) and tomato ketchup (100 mg of lycopene per kilogram).[5] Other sources that have also been found to contain lycopene include rose hips, watermelon, guava, and grapefruit (Figure 10.3).[16] A single feeding of large amounts of lycopene derived from tomato juice has not produced any elevations in the serum.[17] However, 4-week consumption of two to three cans daily of tomato juice has resulted in serum lycopene levels increasing by a factor of 3.

C. Effects of Food Processing

As with most food-processing techniques, there is always a concern for nutrient destruction via heating, ultraviolet light exposure, and mechanical processing. With regard to lycopene, food preparation from cooking results in minimal lycopene losses and its bioavailability and absorption can be further enhanced by the additional ingestion of some dietary fat.[17] When tomato juice was heated in the presence of 1% corn oil, it was observed that a two- to threefold increase in serum lycopene concentration occurred in contrast to no changes in serum concentrations when unprocessed juice was ingested.

Interestingly, lycopene from fresh tomatoes exists predominately in the all-*trans* configuration whereas the human serum and various tissues contain elevated amounts of the *cis*-isomer.[18] However, one investigation that heated lycopene resulted in an increase of the *cis*-lycopene isomer concentration.[17] Therefore, these findings suggest that the *cis*-isomer is more readily absorbed. Similar effects have also been noted with the simple benchtop preparation of spaghetti sauce from canned tomatoes.[19] Like previously described findings, this resulted in higher concentrations of lycopene *cis*-isomers. In a trial conducted by Nguyen and Schwartz,[18] it was reported that tomato products

of varying moisture content, fat content, and container type that underwent thermal treatments resulted in no significant decline in lycopene or changes in the distribution of *cis*-isomers. It was concluded that the abundant levels of *cis*-isomers present in human serum and tissues were not attributed to the ingestion of heat-processed foods but rather due to an *in vivo* mechanism that has yet to be identified.

D. Serum and Tissue Concentration

Given the current data regarding bodily distribution of lycopene as well as other carotenoids, it is already apparent these substances are not homogeneously distributed throughout the tissues and serum of the human body. Furthermore, data already exist which suggest that certain carotenoids are organ specific.[20] In the macula of the human retina, practically the only carotenoids that have been identified are lutein and zeaxanthin. Based on these findings, it might be speculated that these complexes may serve a role in disease prevention of the eye. However, lycopene is a carotenoid that is distributed throughout the serum and a variety of tissues in widely ranging concentrations within the body as well as among different ethnic populations.

Lycopene is the predominant carotenoid found in human plasma.[4] Serum concentrations of lycopene have been found to exist between 0.22 and 1.06 nmol/ml.[21] The serum typically comprises the all-*trans*-isomer as well as many *cis*-isomers. In fact, 12 to 13 isomers have been observed in human serum samples.[22] Almost 50% of the lycopene found in the serum exists as the all-*trans*-isomer. It also accounts for nearly 50% of the total blood carotenoids of individuals in the United States.[9] Lycopene has been reported to be present in an array of human tissues including the testes, adrenal gland, and prostate.[21] Other tissues where it has been located are the liver, skin, lung, and kidney.[21] Typical tissue concentrations reported from a variety of investigations are shown in Figure 10.4. The highest tissue concentration has been reported in the testes and adrenals[23]; however a plausible explanation for these patterns remains uncertain. Human breast milk contains small quantities of lycopene, which warrants further investigation. It has been reported that lycopene from breast milk as well as other carotenoids are present in quantities about 10% of the serum.[24] Breast milk has been reported to contain 34 carotenoids, including 13 geometric isomers and eight metabolites.[25] Two of these metabolites were reported as oxidation products of lycopene. The metabolites were found to contain a novel five-membered ring group and have been identified as epimeric 2,5-cyclolycopene-1,5-diols. Further studies in

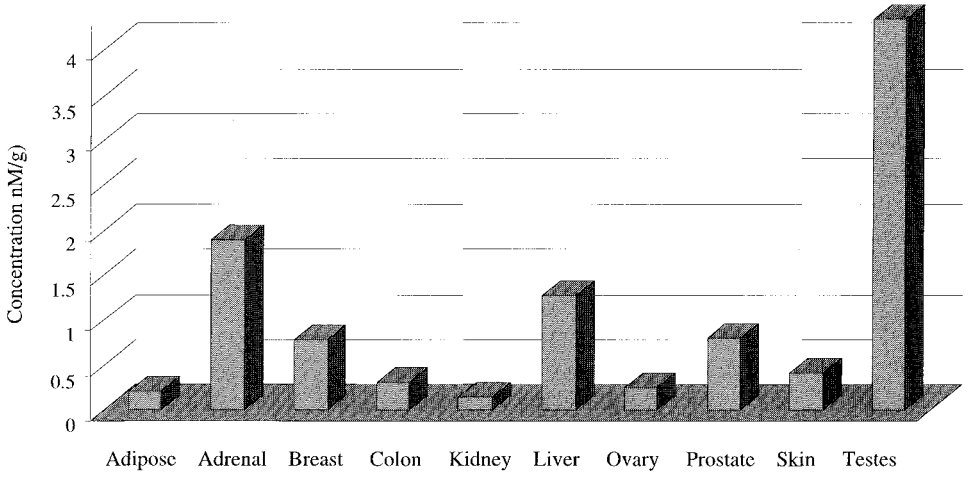

FIGURE 10.4 Concentrations of lycopene found in various tissues.

this area are necessary to determine the relationship of bioavailability of lycopene and its facilitation into the breast-feeding infant.

Gender differences in plasma concentrations have not been reported for lycopene as they have for other carotenoids.[26] Lycopene concentrations have been well correlated to dietary intake, which suggests that there are no differences in absorption or utilization between sexes. Oppositely, in subjects that ingested a low-carotenoid diet for 13 weeks, it was reported that serum lycopene levels declined within the first 2 weeks of the protocol.[27] Based on the data of this investigation, it was estimated that the half-life of lycopene ranges from 12 to 33 days. Of all the carotenoids, lycopene appears to be the exception to the rule with regard to age and plasma concentration. An inverse relationship between age and lycopene concentration has been reported.[26] It is speculated that since dietary lycopene is derived from tomato and tomato products, younger individuals may consume more of these lycopene-rich foods such as pizza and ketchup. Although the oxidants from cigarette smoke have been reported to reduce serum concentrations of β-carotene, similar results have not been produced with respect to lycopene concentrations.[26,28] This is contrasted by a single *in vitro* investigation that reported that various lipophilic antioxidants were reduced as a result of cigarette smoke exposure.[29] With respect to lycopene, it was found to be the most sensitive and was depleted more rapidly than the other lipophilic substances evaluated. The ingestion of alcohol is also a source of oxidative stress which has an effect on various nutrients. However, no impact on serum lycopene concentrations have been reported in those who consume alcohol.[26,28] Another study that supported these findings regarding alcohol consumption and lycopene found that in individuals who consumed 30 g of alcohol per day over 3 months had no changes in lycopene concentrations.[30] However, these investigators found that lutein and zeaxanthin were reduced whereas α- and β-carotene where elevated. It has been reported in patients with alcoholic cirrhosis that lycopene was reduced by as much as 20-fold in the liver.[31] However, it was also reported from this investigation that serum concentrations of lycopene were not reduced.

Interestingly, in one investigation, several demographic factors were associated with lower lycopene serum concentrations.[32] Such factors included geographic location, being unmarried, lower socioeconomic status, and being of nonwhite race. Several dietary factors were also identified in this investigation. The investigators reported that serum lycopene concentrations were reduced in individuals with lower plasma cholesterol, those consuming less vitamin C, and also those having lower dietary lycopene intake.

E. Absorption and Transport

Much of the work regarding carotenoid absorption is based on using β-carotene as a model. It is believed that of the work regarding β-carotene absorption can be applied to lycopene because both are hydrocarbons. In foods, carotenoids, in general, are tightly bound within the food matrix, which may result in various absorption problems and reduce overall bioavailability.[33] There are numerous factors that will be discussed that enhance the initial carotenoid release from the food complex and assimilate the carotenoid into fat droplets within the stomach and small intestines.

Since lycopene is fat soluble, it is absorbed through the same pathway as other lipophilic substances.[4] In order for lycopene to be absorbed, the presence of fat is essential throughout each stage. Therefore, any disease state, drug, or dietary compound that contributes to lipid malabsorption or that disrupts the micelle-mediated process may alter the uptake of lycopene and various other carotenoids. Proper carotenoid absorption may only occur if these compounds are extracted from the food matrix and then incorporated into the lipid phase of the chyme that is present in the gut. It is also important to keep in mind that dietary fat stimulates bile acid secretion, which assists in the formation of lipid micelles. In the presence of fat and bile acids, lycopene is released from food matrices and solubilized in the gut. At the small intestines, ingested lycopene is incorporated into micelles formed from dietary lipids and bile acids. Through passive transport, lycopene is then absorbed into the intestinal mucosa cell. The intact lycopene molecule may then be incorporated

into chylomicrons and will then be subsequently released into the lymphatic system. It seems that the lipoproteins are the only carriers of lycopene as no other carrier or binding proteins have been identified.[34,35] The major vehicle for lycopene as well as the rest of the hydrocarbon carotenoids is the low-density lipoproteins (LDL) fraction.[5] This is in contrast to lutein, zeaxanthin, canthaxanthin, and β-cryptoxanthin, which are oxygenated carotenoids that are more equally distributed among LDL and high-density lipoproteins (HDL).[36] Lycopene initially appears in blood plasma in the chylomicron and very low-density lipoproteins (VLDL) fraction and will then be followed in the LDL and HDL fraction which usually peaks between 24 to 48 h.[5]

It has been suggested that at least 5 to 10 g of dietary fat is necessary to facilitate the absorption of β-carotene.[37] This generally poses no concern since the typical Western diet contributes greater than 30% of its total energy from fat.[38] It has also been suggested that food processing may enhance the absorption of lycopene. It has been reported that lycopene levels in the human serum were increased significantly when processed tomato juice was consumed. This was performed by boiling tomato juice for 1 h in the presence of 1% corn oil.[17] It is believed that the heat processing enhanced the release of the carotenoid by thermally induced cell wall rupture. The presence of corn oil served as a vehicle for enhanced lycopene extraction.

Enhanced absorption of lycopene has been reported when all-*trans*-lycopene and all-*trans*-β-carotene are ingested simultaneously in equal amounts of 60 mg each. Both of which were homogenously dispersed in 1 and 5% corn oil gelatin capsules.[39] Along with the exogenous carotenoids, subjects consumed three low-carotenoid meals, each consisting of 21 g of fat (25% of total energy intake). The results of the investigation indicated that β-carotene significantly improved lycopene absorption, but lycopene did not have any effect on β-carotene absorption. On the other hand, absorption of lycopene as well as the other carotenoids may be inhibited following the ingestion of sucrose polyesters such as olestra. It has been demonstrated that plasma concentrations of lycopene and β-carotene, when ingested simultaneously with 12 g of Olestra for 4 weeks, were reduced by 30 to 50% from baseline.[40] Although this is a significant reduction in these substances, it is believed that the impact due to Olestra will be less serious because snacks containing Olestra are typically not consumed in combination with foods rich in carotenoids. In addition to sucrose polyesters, dietary fibers have also been reported to inhibit absorption of β-carotene, a hydrocarbon similar to lycopene.[41] The absorption of lycopene and other carotenoids were tested after consuming a meal that contained one of the following soluble fibers: pectin, guar, alginate, cellulose, or wheat bran (all consumed at 0.15 g/kg body weight).[42] It was determined that all of these fibers significantly reduced the absorption of lycopene by 40 to 74%. These effects were also noted for β-carotene and lutein.

III. LYCOPENE AND DISEASE

There is a growing amount of data which supports that a diet rich in carotenoids, including lycopene, may serve as a protective agent against various chronic diseases including many types of cancer.[6] A recent publication that reviewed the epidemiological data from 72 studies found that there was an inverse relationship between tomato and tomato product consumption and a reduced cancer risk for 57 of these studies.[43] Of these 57 studies, 35 were found to be statistically significant for the inverse relationship of lycopene or tomato consumption and cancer at a defined anatomical site. The strongest relationships were found for cancers of the prostate, lung, and stomach, whereas lesser relationships were determined for cancers of the cervix, colon, pancreas, esophagus, digestive tract, and breast. Since these are observational studies, no cause–effect relationship can be established. However, as more research is conducted to support these findings, it may be revealed that lycopene may be partly accountable for these health benefits. It is believed that most of the health and biological benefits are purported to occur via protection against oxidative damage.[21] Precedent has already been set that antioxidants found in fruit and vegetables inhibit the oxidation of LDL and help to reduce the risk from coronary heart disease. Lycopene obtained from cooked tomatoes

plays a significant role as an antioxidant that inhibits LDL oxidation.[21] In one aspect of the Rotterdam Study,[44] the serum carotenoid profile of 108 subjects was analyzed in a case-control manner to determine if carotenoids have protective effects against atherosclerosis. After adjustments for age and sex were made, it was determined that lycopene was inversely associated with the risk of atherosclerosis. This finding was not found for any of the other carotenoids analyzed.

Other publications have suggested that diets rich in tomato products may be associated with a reduced risk for prostate cancer.[45,46] In North America, prostate cancer is one of the most prevalent forms of cancers among men. Lycopene has been reported to be present in the prostate in mean concentrations of 0.8 nmol/g.[22] In addition, 14–18 isomers have also been detected in this tissue. However, Americans with prostate cancer have been reported to have 6.2% lower lycopene levels in their blood.[47]

In one prospective study, the relationship between tomato consumption and prostate cancer risk was analyzed.[45] It was reported that the consumption of tomatoes one to four times per week was associated with a lower risk for developing prostate cancer. A larger study that actually analyzed nutrient intake from food frequency questionnaires and the incidence of prostate cancer found that greater lycopene intake was associated with a 21% reduction in risk of prostate cancer.[46] Of the 46 fruit, vegetables, and related products listed in the questionnaire, 4 (tomatoes, pizza, tomato sauce, tomato juice) were significantly related to lower prostate cancer risk. It was estimated the combined intake of tomatoes and tomato products accounted for 82% of the dietary intake of lycopene. The investigators also reported that there was a 35% risk reduction of prostate cancer when the consumption frequency of tomato and tomato products was greater than 10 servings per week compared with 1.5 servings per week. Currently, no mechanism has been identified by which lycopene may reduce prostate cancer risk. Further investigations analyzing lycopene distribution within the prostate and the relationship to other serum carotenoids and dietary intake still needs to be examined. Currently, only a few investigations have evaluated lycopene concentrations in the human prostate.[22,48] In one study,[22] investigators reported that lycopene concentrations in the prostate were observed in concentrations up to 1.8 nM/g. They also reported that the *cis*-isomers of lycopene accounted for 75 to 90% of the prostate lycopene concentration, whereas all-*trans*-lycopene only represented 10 to 25% of the lycopene prostate concentration. In a case-control investigation,[48] serum and tissue concentrations of lycopene and several other carotenoids were measured for 12 subjects. Interestingly, lycopene was the only carotenoid to be reduced in the subjects with prostate cancer. Serum and tissue concentrations of lycopene were 44 and 78% lower, respectively, compared with the matched controls.

Some nonhuman studies have also produced some potentially promising results. One study analyzed the effects of chronic lycopene ingestion and the development of spontaneous mammary tumors in high mammary tumor strains of mice.[49] The results of the investigation suggest that lycopene suppressed mammary tumor development. Similar results were also demonstrated in another study.[50] Lycopene was also reported to display an inhibitory effect on basal endometrial cancer cell proliferation and suppress insulin-like growth factor-I-stimulated growth. As it relates to the insulin-like growth factors, it is important to understand that these factors are the major autocrine and paracrine regulators of mammary and endometrial cancer cell growth.

With regard to cancers of various parts of the gastrointestinal tract, there have been several investigations that reported that lycopene or tomato product consumption reduced the risks of these cancers.[51–54] In one case-control study among Iranian males, it was reported that weekly tomato consumption reduced the risk of esophageal cancer by 40%.[54] In Italy, similar results were also reported for gastric cancer.[53] The investigators reported that diets rich in fruit and vegetables, particularly tomatoes, appeared to be quite protective against this cancer. They found that those who consumed seven servings of tomato products per week were associated with a 50% risk reduction compared with those who only consumed two servings per week. Another study reported that higher serum concentrations of lycopene were associated with a lower risk of gastric cancer.[52]

Research pertaining to lycopene and cervical cancer is still in its infancy and needs further exploration. The link between total fruit and vegetable consumption and risk reduction for various cancers has been established. A few case-control studies of serum concentration or estimated lycopene ingestion have yet to find a significant relationship with risk.[55–57] In one of these studies it was suggested that a small trend for declining serum concentration with cervical disease progression was observed for lycopene and other carotenoids.

The role of lycopene has been briefly evaluated as it relates to skin cancer. It is already known that ultraviolet light may act as photosensitizer which could lead to the formation of free radicals, singlet oxygen, and other highly reactive compounds. Currently, the role of lycopene is unclear with regard to skin cancer risk reduction because conclusive investigations have yet to be performed. However, one study did evaluate the effects of controlled solar-simulated light.[58] In this investigation, women were exposed to ultraviolet light and had their skin lycopene concentrations compared with a nonexposed population. This investigation resulted in a 31 to 46% decrease in skin lycopene concentrations in the ultraviolet light–exposed group. It was also reported that no changes in β-carotene were found, which may suggest that lycopene might have a specific role in defense against ultraviolet light damage. It can further be deduced that diets lacking tomato and tomato products will result in lower serum and skin lycopene concentrations, which may lead to an elevated risk skin damage.

IV. CONCLUSION

At this point in time, dietary supplementation of lycopene does not seem like an appropriate route for improved health. Fruits and vegetables are rich in innumerable chemical compounds including vitamins, minerals, and other biologically active compounds. Furthermore, the consumption of diet abundant in fruits and vegetables typically contains high amounts of fiber and lower amounts of fat. However, keep in mind that small amounts of dietary fat are necessary to facilitate absorption. To date, no recommendations for lycopene intake have been implemented because it has yet to be recognized as a nutrient by any health organizations. Information is also lacking for the potential health risk reductions, mechanisms of action, and dose–response relationships. Additional data also need to be collected to determine the synergistic effect of the phytochemicals present in the diet and how they may protect one from chronic disease.

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11 Cruciferous Vegetables and Cancer Prevention

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I. INTRODUCTION

Diet, particularly a Western diet, is considered to play an adverse role in the etiology of carcinogenesis,[1] as 30% of all cancers are considered to have a dietary component.[2] Numerous purified dietary components have been shown to be mutagenic and are considered by many to be chemical initiators of carcinogenesis,[3] while still other dietary components, such as dietary fat, may act as promoters of carcinogenesis.[4] Fortunately, for we must eat, many diets appear to contain a second group of components that can prevent, slow, or even reverse carcinogenesis. Epidemiological studies have identified that a diet rich in fruits and vegetables is associated with a decreased risk for a number of different cancers.[5,6] Furthermore, the benefit from increasing dietary intake of fruits and vegetables, is not merely due to decreasing the intake of an adverse component, such as dietary fat. Studies specifically evaluating the effect of cruciferous vegetables have shown an inverse relationship between intake of cruciferous vegetables and cancer incidence.[7] One meta-analysis suggests that even as little as 10 g of crucifers/day can have a significant effect on risk.[8] These

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findings have led to a considerable number of clinical and bench studies in an effort to understand and capture this cancer preventative effect for improvement of public health.[9,10]

II. CANCER PREVENTION BY CRUCIFEROUS VEGETABLES

A. Epidemiological Studies

In 1982, the National Research Council on Diet, Nutrition and Cancer found that "there is sufficient epidemiological evidence to suggest that consumption of cruciferous vegetables is associated with a reduction in the incidence of cancer at several sites in humans."[11] A 1996 review of seven cohort studies revealed an inverse association between crucifer ingestion and stomach cancer, cabbage and cauliflower ingestion and lung cancer, and between broccoli ingestion and all cancers.[7] Review of 87 case-control studies indicated that 67% described an inverse association between crucifers and all cancers, with cabbage intake being associated with the greatest number of studies showing this effect.[7] These authors concluded that crucifers decrease the risk for cancer, but this study did not address the relative efficacy of crucifers compared with other fruits and vegetables. More recently a study that evaluated the effect of many individual fruit and vegetables on incidence of bladder cancer among 47,909 men revealed that intake of crucifers, and no other vegetable type examined, was inversely related to risk for bladder cancer.[12] Individually, both broccoli and cabbage had significant effects. Together these epidemiological data strongly suggest that a diet rich in broccoli, cabbage, or a mixture of cruciferous vegetables is able to decrease one's risk for cancer.

B. Laboratory Animal Studies

A number of laboratory animal studies have evaluated the effect of cruciferous vegetables on cancer, mostly by adding powdered, freeze-dried crucifers to the diets of laboratory animals administered chemical carcinogens. The results of these studies support the epidemiological data, suggesting that cruciferous vegetables do protect against carcinogenesis. For example, when broccoli or cabbage was incorporated into the diets of rats that had previously received dimethylbenzanthracene (DMBA), mammary tumor formation was inhibited.[13] Similarly, when rats were given 5 or 10% cabbage following *N*-methylnitrosourea, there was a decreased incidence of mammary cancers.[14] Brussels sprouts (20%) given in the diet before, during, and for 2 weeks after DMBA administration also inhibited mammary tumor formation in rats.[15] Aflatoxin-induced hepatocarcinogenesis was diminished in rats by feeding 25% cabbage[16] and dimethylhydrazine-induced tumorigenesis in mouse was inhibited by a cabbage diet given only during administration of the carcinogen.[17] When mammary tumor cells were placed under the skin of immune-deficient mice, both collard greens and cabbage diminished the appearance of pulmonary metastases.[18] When rats were each given a water extract of Brussels sprouts, or fed 3 g of Brussels sprouts/day, appearance of urinary 8-oxo-guanine as a measure of nitropropane-induced DNA oxidative damage was decreased significantly.[19] These, and many other laboratory studies, support the findings of the epidemiological studies. Something distinctive about cruciferous vegetables permits them to decrease the risk for cancer significantly.

III. CHEMICAL PROFILE OF CRUCIFEROUS VEGETABLES

Evidence from epidemiological studies indicating that crucifers are better able to protect against cancer than many other fruits or vegetables leads to the logical proposal that chemoprevention by cruciferous vegetables is associated with some unique aspect of their biochemistry. Cruciferous vegetables are those belonging to the species *Brassica oleracea*, and include cabbage, Brussels sprouts, cauliflower, broccoli, kale, and collard greens. Cruciferous vegetables contain a series of relatively unique secondary metabolites of amino acids, termed glucosinolates. While glucosinolates are not considered directly bioactive, many glucosinolate hydrolysis products, particularly the

TABLE 11.1
Mean Glucosinolate Content (μmol/g dry mass) in the Edible Tissues of *Brassica oleracea*

Glucosinolates Group	Form	Broccoli	Brussels Sprouts	Cabbage	Cauliflower	Kale
Aliphatics	Sinigrin	0.1	**8.9**	**7.8**	**9.3**	**10.4**
	Gluconapin	1.0	**6.9**	0.7	0.3	1.0
	Glucobrassicanapin	0.3	0.5	0.2	0.1	0.1
	Progoitrin	1.0	**2.4**	0.2	0.3	0.6
	Epiprogoitrin	—	—	—	—	—
	Napoleiferin	0.7	0.4	—	0.2	—
	Glucoiberin	0.1	—	—	—	—
	Glucoraphanin	**7.1**	1.0	0.1	0.5	1.0
	Glucoalysin	0.2	0.1	—	—	—
Phenyl	Gluconasturtiin	0.4	0.5	0.3	0.4	0.4
Indoles	Glucobrassicin	1.1	**3.2**	0.9	1.3	1.2
	4-OH Glucobrassicin	0.2	0.6	0.3		0.1
	4-CH_3OH Glucobrassicin	0.4	0.4	0.3		0.2
	Neoglucobrassicin	0.2	0.2	0.2	0.2	0.1
Total		12.8	25.1	10.9	15.1	15.0

isothiocyanates, appear to have anticarcinogenic activity.[20] Several glucosinolates appear common to all *Brassica*, although their relative abundance varies not only across the vegetable subspecies (Table 11.1), but across varieties within subspecies[21] (Table 11.2), and in response to growing conditions.[22] The range of glucosinolate contents across a group of 50 varieties of broccoli grown under the same conditions varied nearly tenfold from 4.0 to 35.6 μmol/g dry weight.[21] Upon chopping or crushing the vegetable, or even autolysis of the harvested vegetable during storage, glucosinolates come into contact with an hydrolyzing enzyme, myrosinase or thioglucoside glucohydrolase (EC. 3.2.3.1.), located away from the glucosinolate during the life of the plant. The hydrolysis products are glucose, sulfate ions, and an unstable thiono compound (Figure 11.1). The unstable thiono intermediate can rearrange to form an isothiocyanate, a nitrile, or a thiocyanate, depending upon such conditions as pH, temperature, presence of iron, and extent of hydration.[10] The major glucosinolates and their bioactive hydrolysis products from the common *Brassica* vegetables are shown in Table 11.3. A wealth of information is available on the chemistry and bioactivity of glucosinolate hydrolysis products.[9,10,22]

Crucifers have a high fiber content, about 30% of their dry matter, with the aboveground portion of the plant being approximately 70 to 90% water by weight, depending upon the tissue. Crucifers are also rich sources of a number of vitamins, including several carotenoids (β-carotene, lutein, zeaxanthin), vitamins C, E, and K,[23] as well as sterols.[24] When 34 fruits and vegetables commonly consumed in Sweden were compared, broccoli, Brussels sprouts, and cauliflower had the highest sterol content, ~50 mg/100 g portion. These characteristics are shared to a greater or lesser degree by many other fruit and vegetables, and are therefore unlikely to be the major reason for a crucifer-specific anticancer effect, although they might play a supporting role, enhancing effects of glucosinolate hydrolysis products. Crucifers contain quite high concentrations of *S*-methyl cysteine sulfoxide which can break down to release volatile sulfur compounds such as thiols, dimethyl disulfide, and dimethyltrisulfide, compounds associated with a musty odor during cooking and an unpalatable flavor.[25] Calorie restriction has been shown to alter detoxification enzymes and lower risk for cancer.[26] Therefore, depressed feed intake due to lack of palatability may be a confounding influence in anticancer studies. Even if control and experimental animals consume the same weight of food, because of the high fiber content in crucifers, it is important that control and experimental diets are balanced for calories and nutrients,[27] particularly when animals are fed 20 or even 30% of their diet as vegetable powder.

TABLE 11.2
Glucosinolate Content (μmol/g dry weight) of Selected Accessions of Broccoli

	Aliphatics					Indoles			Phenyl
Accession	Sinigrin	Progoitrin	Glucoraphanin	Residual	Total	Glucobrassicin	Residual	Total	Gluconasturtiin
Brigidier	0.0	0.9	21.7	3.8	26.3	1.2	1.0	2.2	0.7
Eu 8-1	0.1	7.9	9.6	3.3	20.9	1.3	1.0	2.3	0.6
Florette	0.0	0.3	8.7	1.5	10.4	1.0	0.5	1.6	0.6
Majestic	0.0	0.1	16.0	3.0	19.1	0.3	0.4	0.7	0.2
Peto no.7	0.1	7.2	5.9	18.3	31.4	1.2	2.4	3.6	0.6
Pirate F1	0.0	0.5	10.7	1.9	13.0	2.8	1.3	4.1	0.6
Shogun	0.8	2.6	11.9	3.3	18.7	2.4	3.8	6.2	0.9
VI 158 DH I.S.	6.0	5.3	9.4	10.5	31.2	1.3	0.6	1.9	0.5
Wintergarden	0.0	0.5	17.5	0.9	18.9	1.6	0.9	2.5	0.5
Zeus	0.0	0.1	2.9	0.8	3.8	1.1	1.3	2.3	0.3

TABLE 11.3
Major Glucosinolates and Bioactive Hydrolysis Products in *Brassica oleracea*

Major Glucosinolates	Bioactive Hydrolysis Products
Glucobrassicin	Indole-3-carbinol
Gluconasturtiin	Phenylethyl isothiocyanate
Glucoraphanin	Sulforaphane
Progoitrin	Crambene
Sinigrin	Allyl isothiocyanate
Glucotropaeolin	Benzyl isothiocyanate

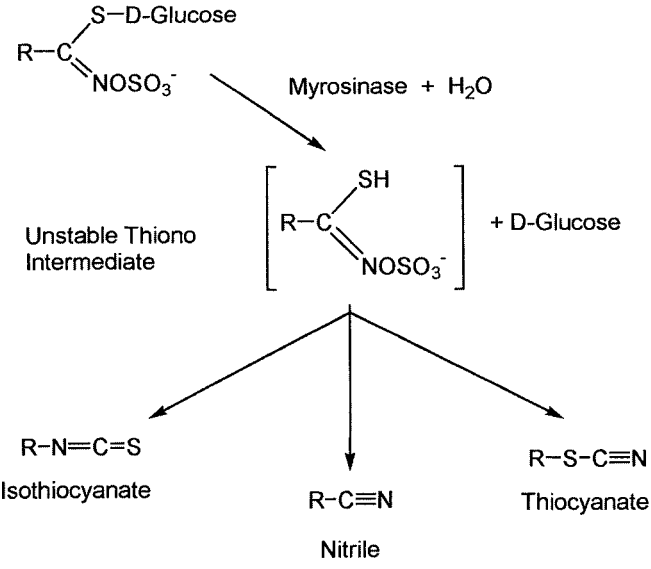

FIGURE 11.1 Myrosinase-dependent hydrolysis of glucosinolates.

IV. MECHANISMS OF CHEMOPREVENTION

In seminal studies planned to determine the mechanism of cancer prevention by isothiocyanates, Wattenberg treated rats with a single dose of benzyl isothiocyanate, either 24, 4, or 2 h before, or 4 h after, giving the carcinogen DMBA.[28] He found no effect on tumor incidence when benzyl isothiocyanate was given 24 h before or 4 h after the carcinogen. Treatment with benzyl isothiocyanate 4 h before exposure to the carcinogen was significantly less effective than 2 h prior to the carcinogen, which decreased the incidence of mammary tumors by 77%.[28] This response indicated that even a single dose of benzyl isothiocyanate could prevent initiation, within a narrow window of time. Interestingly, when he administered benzyl isothiocyanate in the diet starting 1 week after DMBA treatment, fewer breast tumors developed.[29] This he interpreted as indicative of a protective role for benzyl isothiocyanate during promotion. Since these early studies, it has become increasingly clear that the anticarcinogenic effects of isothiocyanates are unlikely to be due to a single mechanism, or even limited to a single stage of carcinogenesis. While the research supporting a role for isothiocyanates as anticarcinogens is legion, it is not yet clear the extent to which these are responsible for the anticarcinogenic effect of the whole vegetable, or whether other bioactive components play an equally effective role.

A. Induction of Detoxification

The major mechanism of chemoprevention by cruciferous vegetables is thought to be through upregulation of detoxification enzymes, resulting in decreased initiation of chemical-induced carcinogenesis.[30] First proposed 25 years ago, this theory has undergone a number of refinements,[31] but remains essentially unchanged. Clinical studies have shown clearly that ingestion of cooked cabbage and Brussels sprouts daily for 10 days significantly increases the clearance of drugs in healthy college students, and that this effect is reversible given a diet free of cruciferous vegetables.[32] Similarly, 500 g of broccoli/day for 12 days significantly increased cytochrome P450 (CYP)1A2-dependent caffeine metabolism, even though it did not alter CYP2E1-dependent chlorzoxazone metabolism.[33] A diet containing 300 g of cooked Brussels sprouts/day for 3 weeks increased serum α-class glutathione transferase 1.4-fold.[34] Together these data indicate that dietary

crucifers can increase detoxification enzymes in humans. Rodent feeding studies confirming this effect of whole cruciferous vegetables[35-37] show that specific detoxification enzymes are upregulated by dietary crucifers.

Many aliphatic glucosinolate hydrolysis products cause an upregulation of several phase II detoxification enzymes, including quinone reductase and several glutathione-*S*-transferases. Indole-3-carbinol, derived from the indolylic glucosinolate glucobrassicin, causes an upregulation of both these phase II enzymes and the phase I cytochrome P450 enzymes CYP1A1/2. Some concerns have been raised about the safety of CYP1A1 induction, since it not only detoxifies a number of xenobiotics, but also activates a number of precarcinogens, including many polycyclic hydrocarbons.[38] Because of this, it has been suggested that monofunctional inducers, which increase only the phase II enzymes, may well be more effective as chemopreventative agents than the indoles, termed bifunctional because of their ability to induce both phase I and phase II enzymes.[39] This is an interesting hypothesis, given that animal studies evaluating the anticarcinogenicity of indole-3-carbinol have mixed results. Alternatively, the beneficial effects of indole-3-carbinol on phase II metabolism may overwhelm any adverse effects associated with stimulation of phase I enzymes. All *Brassica* vegetables contain glucobrassicin, the parent glucosinolate of indole-3-carbinol, and both epidemiological and laboratory animal studies have shown that the whole vegetable protects against cancer. If indole-3-carbinol causes deleterious as well as anticarcinogenic responses *in vivo*, then additional components found in the whole vegetable or modifications in metabolism of indoles from the whole vegetable appear capable of neutralizing the harmful effects.

Designation of compounds as mono- or bifunctional inducers is useful in considering mechanism of induction of detoxification enzymes. On a molecular level, glucosinolate hydrolysis products upregulate through at least two response elements in the genes of detoxification enzymes: (1) bifunctional inducers interact with the xenobiotic response element (XRE), also termed the aryl hydrocarbon response element (AhRE) and (2) monofunctional inducers interact with the antioxidant response element (ARE). The XRE is found in the DNA regulatory region of the genes for a number of proteins, including CYP1A1/2, quinone reductase, and glutathione transferase Ya2.[40] The ARE is found in the regulatory regions of the genes for quinone reductase and glutathione transferase Ya2, but not CYP1A1/2.[41] Thus, gene sequences for these phase II enzymes contain regulatory regions with both an XRE and an ARE, while gene sequences for CYP1A1/2 lack the ARE and lack the ability to be stimulated by monofunctional inducers. In addition, the regulatory sequence in the gene for γ-glutamyl cysteinyl synthase, the rate-limiting step in the synthesis of glutathione, contains several ARE sequences,[42] but no XRE has been identified. The classification of molecules that upregulate enzymes as monofunctional or bifunctional inducing agents is therefore more closely aligned with whether the ARE (monfunctional) or the XRE (bifunctional) is triggered, than it is with describing the full range of interactions between glucosinolate hydrolysis products and the entire family of CYP enzymes. While regulatory regions of several other genes have been found to include one or both of these DNA sequences, no exhaustive search of the genome has been conducted.

B. Inhibition of Activation

A large number of precarcinogens are bioactivated to the ultimate carcinogen by CYP-dependent oxidation.[38] It has been proposed that glucosinolate breakdown products may protect against initiation of cancer not only by induction of phase II detoxification enzymes, but also by inhibiting CYP-dependent activation of precarcinogens.[43] The anticarcinogenic action of isothiocyanates against nitrosamines has been proposed to be due to inhibition of bioactivation of the nitrosamines, a CYP2E1-dependent activity.[44] Both phenylethyl isothiocyanate (PEITC)[45] and sulforaphane[46] have been found to inhibit the phase I enzyme, CYP2E1. When a group of arylalkyl isothiocyanates were evaluated as inhibitors of DNA adduct formation, several were found to be more effective than PEITC, and their ability to inhibit tumorigenesis correlated well with their inhibitory effects

on DNA adduct formation. This suggested to the authors that the most likely mechanism was via inhibition of CYP-dependent bioactivation.[47] Very recently benzyl isothiocyanate was shown to cause the destruction of CYP2E1 during metabolism.[48] This type of "suicide" inhibition is far more effective *in vivo* than are competitive inhibitors, and could be expected to inhibit any CYP2E1 metabolism substantially, even at very low levels. Add to this inhibitory effect on CYP2E1, the ability of benzyl isothiocyanate to activate phase II enzymes and one could have a very potent anticarcinogenic compound. It remains to be seen if all isothiocyanates inhibit CYP2E1 in the same manner, or if benzyl isothiocyanate is alone in causing destruction of the CYP2E1.

When other enzyme activities were examined, PEITC but not sulforaphane or allyl isothiocyanate was found to inhibit rat microsomal CYP1A1/2 and CYP2B1/2,[49] in addition to CYP2E1. This study did not evaluate the mechanism, so one cannot determine the type of inhibition, and whether it would be likely to have an effect *in vivo*. In a temporal study using rats, PEITC was found to cause a significant loss of CYP1A1/2 and CYP3A activity, in addition to the severe loss of CYP2E1 activity.[45] In contrast to the *in vitro* study that had reported acute inhibition of CYP2B enzyme activity, here PEITC was found to induce CYP2B enzymes. These data show that not only do the glucosinolate hydrolysis products have multiple effects on the CYP enzymes, but that results may vary substantially between *in vitro* and *in vivo* effects, between acute, subacute, and chronic effects. It is important to choose models that correctly reflect conditions expected when one eats cruciferous vegetables intermittently, as most consumers do. It would be of use to know if one should include crucifers in the daily diet to get any benefit, or whether inclusion every few days would be sufficient to maintain the effect. Other drug-metabolizing enzymes reported to be inhibited by glucosinolate hydrolysis products include flavin monooxygenase[50] and aldehyde dehydrogenase.[51] However, the possible role that their inhibition might play in cancer prevention has yet to be evaluated.

C. Inhibition of Cell Proliferation and Apoptosis

Another mechanism whereby cancer may be prevented is through a decrease in number, or inhibition of growth, of cells that have been transformed to a malignant state. The idea that proliferation of cancer cells may be preferentially inhibited is the basis for attempts to identify compounds that slow or stop tumor growth. To test the hypothesis that glucosinolates may work through this mechanism, cells in culture were treated with parent glucosinolates and their effects on cell number and cell cycle were analyzed. In general, the parent glucosinolates had little or no effect on cells in culture until toxic levels were reached. In one study, nine glucosinolates from Brassica seeds were without effect on the growth of erythroleukemia K562 cells. By contrast, after myrosinase-dependent hydrolysis, most of the glucosinolate hydrolysis products were capable of inhibiting proliferation. Unfortunately, many of the common glucosinolates in dietary crucifers, including glucobrassicin, gluconasturtiin, and glucoraphanin, were not evaluated in this study.[52] In a separate study using the human undifferentiated colon cancer cell line HT29, the parent glucosinolate, glucobrassicin, was shown to have no effect (100 μM), while the hydrolysis products diindolyl methane (10 μM) and sulforaphane (20 μM) decreased cell viability by approximately 50%.[53]

An important consideration in studies of tumor cell cytotoxicity is whether tumor cells are selectively more sensitive than untransformed cells. In one study, a canine breast cancer cell line was found to be more sensitive to the cytotoxic effects of the cruciferous nitrile crambene (in the presence of selenium) than were untransformed, primary canine mammary cells.[54] Others have shown that human colon cancer cell lines are more sensitive to sulforaphane (CaCo2 cells) or allyl isothiocyanate (HT29 cells) during proliferation than following confluence, when the cells become differentiated and more closely resembled normal, untransformed cells.[53,55]

Gamet-Payrastre and co-workers[53] associated the antiproliferative effects of diindolyl methane and sulforaphane in HT29 cells with cell cycle arrest in G0/G1, preventing reentry into the cell cycle and synthesis of DNA. Other studies have also implicated cell cycle arrest. A study

in HeLa cells reported that allyl isothiocyanate, benzyl isothiocyanate, and PEITC all caused cell cycle arrest at G2/M.[56] Similarly, a study in HepG2 cells showed G2/M arrest following treatment with the nitrile crambene (A.S. Keck, personal communication). Some isothiocyanates may arrest growth at G0/G1, while others cause G2/M arrest. However, differences reported here could also be due to differences between studies, including experimental design and cell type. It would be interesting to evaluate these compounds in a single system, allowing effects on cell cycle and proliferation to be compared directly.

Tumor formation and growth may be controlled through a decrease in cell proliferation via arrest of the cell cycle and/or an increase in apoptosis or programmed cell death. Oral administration of the glucosinolate sinigrin induced apoptosis and significantly decreased the formation of aberrant crypt foci in colon of rats exposed to dimethylhydrazine.[57] The authors suggested that apoptotic deletion of damaged stem cells in sinigrin-treated rats could be the cause for decreased formation of aberrant crypts. In addition to this *in vivo* study, isothiocyanates have been found to cause apoptosis *in vitro*. In a mechanistic study, Yu and co-workers[58] reported that isothiocyanates caused apoptosis in HeLa cells. This isothiocyanate-induced apoptosis was associated with an increase in caspase 3, a proteolytic enzyme in the pathway of programmed cell death. Interestingly, not only was caspase 3 increased prior to apoptosis, but an inhibitor of caspase 3 was able to inhibit apoptosis in the presence of isothiocyanate, suggesting that isothiocyanate-dependent apoptosis is therefore mediated through caspase 3. c-Jun N-terminal kinase (JNK), which lies upstream of caspase 3 in the apoptotic cascade, was also activated by isothiocyanates. When JNK activation was blocked, isothiocyanate-induced apoptosis was also suppressed, indicating that JNK has a role in mediating isothiocyanate-induced apoptosis.[59] In work from another group of researchers, PEITC was found to induce apoptosis through a p53-dependent pathway, leading these authors to suggest that PEITC-dependent JNK activation may increase p53 phosphorylation.[60] Additionally, overexpression of Bcl-2, which typically opposes Bax, was found to suppress PEITC-induced JNK activation and block PEITC-induced apoptosis. Together these data support the hypothesis that, through JNK activation, PEITC may lead, via p-53, Bax, and caspase 3, to destruction of tumor cells by apoptosis. Studies of this nature, which continue to trace the effects upstream, add considerably to our knowledge of how isothiocyanates affect tumor growth and programmed cell death. There may be additional stages in the cell cycle where isothiocyanates affect apoptosis, and where molecular studies can help clarify the complex interactions of these signaling molecules.

V. DEVELOPMENT OF CANCER PREVENTATIVE AGENTS FROM CRUCIFEROUS VEGETABLES

A large volume of information has accumulated on the cancer preventative activity of isothiocyanates derived from hydrolysis of glucosinolates.[20,22,61,62] The majority of the work has focused on just a few, including indole-3-carbinol, PEITC, sulforaphane, and benzyl isothiocyanate. Scattered studies have shown protection from other indolyl compounds, such as brassinin, which has been shown to protect against mammary carcinoma and skin tumors in mice given DMBA,[63] and allyl isothiocyanate, effective in combating colon carcinogenesis.[64] In structure–activity studies, a series of arylalkyl and alkyl isothiocyanates were compared for their ability to protect against carcinogenesis. Several synthetic secondary isothiocyanates were found more effective than PEITC,[65] with increasing chain length leading to increasing efficacy in prevention of NNK-induced tumorigenicity.[66] Yet when a similar series of compounds were evaluated for anticarcinogenesis in two models of carcinogenicity, no consistent order of potency was seen across the two models.[47] Interestingly, potency was correlated to inhibition of DNA-adduct formation across both models. It appears unlikely that research will identify a "magic bullet," natural or synthetic, with the ability to quench all cancers. Two glucosinolate breakdown products, PEITC and indole-3-carbinol, are being developed as prophylactic drugs. These purified compounds may eventually be routinely administered

to persons at high risk for cancer. In other work, researchers are using the potent isothiocyanate sulforaphane as the starting point for development of similar synthetic anticarcinogenic agents. Bioactivity of nonglucosinolate components has been less well studied.

A. Phenylethyl Isothiocyanate

Watercress is a particularly good source of gluconasturtiin, the parent glucosinolate to PEITC.[22] Feeding watercress to smokers blocked the metabolic activation of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), a major carcinogen in tobacco.[67] In an evaluation of the effect of PEITC on NNK bioactivation in rats, it was found that PEITC (1 mmol/kg rat) had an inhibitory effect on CYP2E1, effectively blocking the bioactivation of NNK.[45] Also, PEITC induced a number of phase II enzymes which could then clear any bioactivated products that were formed.[45] Rodent studies have shown clearly that PEITC inhibits NNK pulmonary carcinogenesis when given before or during NNK administration.[68] Thus, PEITC not only inhibits NNK-induced lung tumorigenesis in rats and mice, but alters rodent metabolism of NNK in the same way that feeding watercress altered the urinary metabolites in smokers. These data have been used to support the suggestion that PEITC, or PEITC-rich vegetables, could be used as chemopreventative agents in smokers to delay or forestall the onset of lung cancer.[61]

When PEITC administration to rats was commenced 1 week after NNK, no antitumorigenic effect was seen, even though it was given continuously for 15 weeks.[69] This observation suggests that while PEITC might block initiation, it is not able to suppress proliferation. One study reports that, when PEITC and benzyl isothiocyanate were given postinitiation to rats that had received the urinary bladder carcinogen diethylnitrosamine, there was a significant increase in papillary hyperplasia and carcinoma compared with rats receiving the carcinogen alone. In addition, there were papillomas and carcinomas in a few rats that received isothiocyanates and no carcinogen.[70] The authors concluded that these isothiocyanate are strong promoters of urinary bladder carcinogenesis and even have some complete carcinogenic potential, both initiating and promoting tumor growth. Although this information comes from only one study, compared with the many studies that suggest a positive benefit from PEITC, it serves as a good warning that information regarding toxicity and carcinogenicity is needed before purified plant secondary metabolites are used as drugs, regardless of the food quality of the plant source.

B. Indole-3-Carbinol

There are more reports on indole-3-carbinol, its chemistry, toxicology, and potential efficacy as an anticarcinogenic agent, than on any other glucosinolate hydrolysis product.[71,72] In animal studies, indole-3-carbinol has been found to inhibit the development of tumors in the forestomach,[73] stomach,[74] mammary gland,[14,75] uterus,[76] tongue,[77] and liver[78] of rodents, and in the liver of trout[79] when administered prior to, or during, carcinogen exposure. Because of the many positive anti-initiation studies in laboratory experiments, indole-3-carbinol, like PEITC, has been carried into clinical evaluation as a anticarcinogen in individuals at high risk for cancer. Clinical trials evaluating long-term indole-3-carbinol treatment in patients with recurrent respiratory papilloma have found that, of 18 patients administered oral indole-3-carbinol, 6 exhibited cessation of papilloma growth and 6 exhibited significantly reduced papilloma growth. No adverse effects were reported over more than 8 months of treatment.[80] A breast cancer prevention trial is being conducted at Strang Cancer Center, based on mechanistic studies showing that in three breast cancer cell lines, indole-3-carbinol caused a >50% inhibition in growth, with an increase in the quiescent cell fraction (G0/G1 phase of the cell cycle), and a doubling of the apoptotic rate.[81] Although this suggests that the use of indole-3-carbinol is extremely promising, there are also concerns over its potential use as an anticarcinogenic agent, questioning whether, under certain conditions, indole-3-carbinol might enhance tumorigenicity.[82]

At first glance, that enhancement of cancer is possible appears predictable. Indole-3-carbinol condensation products have the ability to bind to the XRE and cause induction of CYP1A1/2, which is involved in the bioactivation of many precarcinogens. Rats fed 100 mg indole-3-carbinol/kg for 5 days showed a 14-fold increase in ethoxyresorufin *O*-deethylase, a measure of CYP1A1/2 activity,[83] and even 50 mg/kg diet (~2 or 3 mg/kg rat) caused a significant induction of CYP1A1/2 activity.[84] When indole-3-carbinol was administered to mice prior to the pulmonary carcinogen NNK, pulmonary NNK-DNA adduct formation was decreased, but hepatic NNK-DNA adduct formation was increased. The authors concluded that indole-3-carbinol had merely decreased distribution of NNK to the lung, by enhancing bioactivation in the liver.[85]

However, most feeding studies that report enhancement of tumor formation by indole-3-carbinol do not focus on a role in activation of the precarcinogen, but rather in promotion. Exposure to indole-3-carbinol postinitiation has been associated with enhanced tumor formation in liver of trout and rats,[79,86] in colon of rats and mice,[87] in thyroid of rats,[86] and in pancreas of hamsters.[88] Administration to rats daily for 24 weeks caused a significant increase in liver weight,[86] suggesting that, like phenobarbital, indole-3-carbinol may be mitogenic when given on a daily basis. It would be interesting to know how indole-3-carbinol affects promotion if given less often than daily. There might be a dosing regimen that could maintain upregulation of detoxification enzymes, without supporting mitogenesis. Also, it is worth remembering that glucobrassicin is present, to various extents, in all *Brassica* vegetables. Therefore all the epidemiological data showing a decreased risk for cancer are based on individuals ingesting glucobrassicin or its hydrolysis product, indole-3-carbinol, in addition to other glucosinolates and their derivatives.

Indole-3-carbinol is not a simple chemical to study, for many active compounds are derived from it. Under the acid conditions of the stomach, indole-3-carbinol undergoes condensation to form multiple complexes.[89] Some of the products, most particularly diindolyl methane and indole-3-carbazole, are effective ligands for the cytosolic Ah receptor. Upon binding to the receptor, the receptor–ligand complex is transported to the nucleus and can interact with the xenobiotic response element (XRE) of a variety of genes, including CYP1A1/2, triggering bifunctional induction of detoxification enzymes. Although some of the condensation products are Ah receptor agonists, others may be partial agonists or even antagonists, capable of binding the Ah receptor, but preventing activation of the XRE.[89] In contrast, indole-3-carbinol itself has poor affinity for the Ah receptor and results in little or no upregulation of CYP1A1/2. However, indole-3-carbinol is not without activity, since it interacts with the ARE to cause weak monofunctional induction (C.-W. Nho, personal communication). Many *in vitro* studies have evaluated the individual effects of diindolyl methane and indole-3-carbazole, but there are almost no *in vivo* studies of the effect that these condensation products have during the promotion phase of carcinogenesis. One study in which rats were treated with diindolylmethane following DMBA reported a decrease in mammary tumor growth without an induction of CYP1A1.[90]

Indole-3-carbinol and at least some of these condensation products have an interactive effect with estrogen, affecting estrogen metabolism and estrogen binding to the estrogen response element.[91,92] A number of studies in rodents and humans show that indole-3-carbinol causes an increase in 2-hydroxylation of estrogen, increasing the ratio of 2:16 hydroxylated products. For example, men and women, given 400 mg indole-3-carbinol daily for 1 week, exhibited an increase in the 2-hydroxylation of estrogen.[93] Clinical use of indole-3-carbinol as an anticarcinogen is based partly on the strategy that estrogen-dependent tumors will not be supported by this altered environment, since unlike 16-hydroxylated products, 2-hydroxylated products are not estrogenic. In confirmation of this theory, when human breast cells were grown in medium containing indole-3-carbinol, the increase in the ratio of 2:16 hydoxylated estrogens was inversely proportional to the growth of the cells.[81] Clinical studies evaluating the effect of both indole-3-carbinol and whole crucifers show an inverse relationship between the extent to which the 2:16 ratio of hydroxylated products is increased, and tumor growth.[80,94] More recent *in vitro* data suggest that antiestrogenic effects of diindolyl methane are seen at doses at least an order of magnitude less than those necessary to

produce an observable change in estrogen 2-hydroxylation. While the mechanism of antiestrogenicity is still under investigation, this effect appears to be Ah-receptor dependent.[90]

C. Sulforaphane and Sulforaphane Analogues

Of a large number of glucosinolate hydrolysis products tested for their ability to upregulate quinone reductase in cell culture, sulforaphane was found to be one of the most potent.[95] In addition, sulforaphane decreases mammary tumor incidence when administered to rats[20] before and during administration of DMBA, and inhibits neoplastic nodule formation in mouse mammary gland cultures.[96] Sulforaphane, in low μM concentrations, inhibits DNA damage caused by mutagens that must be bioactivated by CYP2E1 and CYP1A2, providing the possibility that inhibition of the CYP may contribute to the chemopreventative action of sulforaphane.[46] The high potency of sulforaphane, <0.6 μM causing a doubling of quinone reductase in mouse hepatocyte culture,[95] has sparked an interest in the synthesis of similar compounds.[97] Synthesis of 35 structural analogues revealed that the most potent compounds were those with either a methylsulfonyl group (like sulforaphane) or an acetyl group 3 to 4 carbons distant from the isothiocyanate moiety. The ketoisothiocyanate (+/–)-exo-2-acetyl-6-isothiocyanatonorbornane, or compound 30, was found to be an equipotent agent to sulforaphane both *in vivo* and *in vitro* for its effect on quinone reductase levels. When sulforaphane and compound 30 were administered to rats given DMBA, they were both effective as anticarcinogens.[20] Possible advantages of compound 30 over sulforaphane are that it is easily synthesized and relatively more stable than sulforaphane. A second group of researchers has developed a sulforaphane analogue 4-methylsulfinyl-1-(*S*-methyldithiocarbamyl)-butane, or sulforamate. This compound was also found to be equipotent to sulforaphane in induction of quinone reductase *in vitro* and in inhibition of preneoplastic lesion formation in a carcinogen-treated mouse mammary gland organ culture.[96] In addition, sulforamate was found to be threefold less cytotoxic that sulforaphane, possibly suggesting a greater margin of safety if used as a prophylactic drug in individuals at high risk for cancer. It is interesting to note that these attempts to synthesize new analogues produced no major improvements in potency over the natural product, sulforaphane. However, they are more stable, and stability must be of concern both in fresh vegetables where sulforaphane is seen to withstand storage very poorly[98] and in tablets of sulforaphane or broccoli that have no shelf life or expiration dates on the label.

VII. BIOACTIVE COMPONENTS OTHER THAN ISOTHIOCYANATES

Studies that compare the effects of whole cruciferous vegetables with those of purified glucosinolates and/or hydrolysis products do not completely support the hypothesis that glucosinolate hydrolysis products are solely responsible for the upregulation of detoxification enzymes. McDanell and co-workers[99] fed Brussels sprouts, a glucosinolate extract, or pure glucobrassicin to rats and estimated ethoxyresorufin *O*-deethylase activity. They found that the extract not only contained less glucobrassicin, but was less effective than the whole vegetable. Although the decreased effect could be explained by a dose–response to glucobrassicin, pure glucobrassicin, hydrolyzed or unhydrolyzed, was severalfold less potent than the whole vegetable. Similarly, in a comparison of the induction of hepatic glutathione transferases produced by feeding Brussels sprouts powder, or by giving pure glucosinolate hydrolysis products to rats, the whole vegetable appeared many times more potent than comparable quantities of purified glucosinolate hydrolysis products.[36] Studies of this type have caused several researchers to question whether there are more bioactive components than just isothiocyanates in cruciferous vegetables.

Two approaches have been used to evaluate this possibility: fractionation of the whole vegetable and purification and testing of individual candidate nonisothiocyanate compounds. Fractionation of broccoli has led Talalay and colleagues to suggest that, at least in broccoli, sulforaphane accounts for essentially all the upregulation of detoxification enzymes.[95] Fractionation of Brussels sprouts,

vegetables with a more complex glucosinolate makeup than many broccoli varieties (see Table 11.2), suggests that other components may play a major role in anticarcinogenesis and upregulation of detoxification enzymes.[100] At this time, there are no reports in the literature of the effect of feeding rats increasing quantities of a cruciferous vegetable, while keeping the glucosinolate level unchanged. Some of the nonisothiocyanate compounds that have been studied for bioactivity in their pure form are crambene, *S*-methyl cysteine sulfoxide, (SMCSO) and dithiolethione.

A. Crambene

In evaluation of glucosinolate hydrolysis products as anticarcinogens, almost all the research has focused on isothiocyanates. Most nitriles are relatively toxic, but fortunately are also relatively unstable. One nitrile, crambene (1-cyano-2-hydroxy-3,4-butene) is both stable and bioactive. Although somewhat toxic at doses greater than 100 mg/kg rat, producing hepatic and pancreatic apoptosis, doses of 30 mg/kg rat were associated with upregulation of glutathione synthesis and no toxicity.[100] A feeding trial has identified that 1 g/kg diet given to rats before, during, and after aflatoxin significantly decreased the number of γ-glutamyl transpeptidase-positive foci (a measure of precancerous cells) at 12 weeks after aflatoxin.[101] Further studies have revealed that crambene is a monofunctional inducer, causing upregulation of quinone reductase and glutathione transferase while having no effect on CYP1A1/2 levels.[102] Interestingly, when rats were given both crambene and indole-3-carbinol together, total upregulation of quinone reductase was significantly greater than expected when adding together their individual effects.[102] These data suggest that the combined bioactive products in cruciferous vegetables may interact and be more effective than each is individually. Two implications follow from this finding. First, the effective dose of the vegetable, which contains a mixture, may be smaller than originally calculated from efficacy of individual glucosinolate hydrolysis products. Second, two or three bioactive components, while providing additive if not synergistic efficacy, may have individual toxicities that are not additive. Interactions deserve a more thorough evaluation.

B. *S*-Methyl Cysteine Sulfoxide

The odor of crucifers is, like the onion family, mostly associated with sulfur compounds. Crucifers contain high levels of SMCSO, a compound that gives rise to several sulfides not unlike those found in garlic, and with a similar toxicology at very high levels of intake.[103] When rats were administered a diet containing 4% SMCSO, they exhibited anemia and splenic hypertrophy, which reversed upon removal of SMCSO from the diet.[104] These experimental doses of SMCSO are far greater than those normally contained in cruciferous vegetables. Brussels sprouts contain <0.1% SMCSO (0.5 mmol/100 g fresh tissue) which is about three- to fivefold greater than in other brassica. When mice were administered doses of SMCSO that were comparable to those observed in brassica (0.5 mmol SMCSO/kg body weight), genotoxicity of benzo[a]pyrene was decreased by one third.[105] The possibility that SMCSO may be responsible in part for the anticarcinogenic effect of cruciferous vegetables deserves further attention.[106]

C. Dithiolethione

1,2-Dithiole-3-thione is the natural dithiolethione present in cruciferous vegetables. It has, however, been studied less thoroughly than a related synthetic compound, 5-(2-pyrazinyl)-4-methyl-1,2-dithiole-3-thione, known as oltipraz. Oltipraz is an antischistosomal drug which has been found effective as an anticarcinogen in a number of animal studies.[107] Both dithiolethione and oltipraz have been shown to upregulate phase II enzymes via the ARE,[108] making them classic monofunctional inducers. Oltipraz has been taken into clinical trial, where 125 mg/m^2, the smallest dose evaluated, caused an increase in colonic glutathione-S-transferase and quinone reductase.[109] No adverse effects were seen in patients taking this dose twice weekly for 12 weeks.

Effects of oltipraz on detoxification have also been evaluated in a clinical study. Residents of Qidong province in China have a diet that is particularly contaminated by aflatoxins, and are seen to have an associated high risk for liver cancer. Healthy adults (234) were divided into three groups and given either a placebo, 500 mg oltipraz once weekly, or 125 mg oltipraz daily. After 1 month, urinary analysis revealed that the intermittent high dose inhibited phase I bioactivation of aflatoxin, while daily low-dose oltipraz increased phase II detoxification of bioactivated aflatoxin.[110] These are particularly exciting results when compared with animal studies. Not only does oltipraz alter aflatoxin metabolism and protect against aflatoxin-induced liver cancer, but the unsubstituted dithiolethione found in cruciferous vegetables is more potent than oltipraz in both cell culture studies evaluating induction of detoxification enzymes, and in whole rat studies evaluating protection against aflatoxin-induced liver tumors.[111]

VIII. SAFETY OF CRUCIFEROUS VEGETABLES

The toxicology of glucosinolate-containing plants, both cruciferous vegetables and oilseed crops such as crambe and rape, has been reviewed in detail elsewhere.[22] Using a differential DNA repair assay to evaluate the genotoxicity of vegetable extracts, potency was found to vary across the subspecies with Brussels sprouts > cauliflower > cabbage, kohlrabi, broccoli > turnip, black radish.[112] This ranking was not strictly related to total isothiocyanate content, but the authors suggested that this might only be because individual isothiocyanates differ in their genotoxic potency. In one study, PEITC and benzyl isothiocyanate were found to act as promoters of chemical-induced urinary bladder papillary carcinoma, even in control rats receiving no carcinogen. This study suggested that these isothiocyanates were both promoters and complete carcinogens, initiating cancer and promoting mitosis of mutant cells.[70] An added concern is the fact that if cruciferous vegetables are exposed to nitrate or nitrite under acid conditions, such as ingestion of unwashed fresh vegetables contaminated by nitrate fertilizers, or ingestion of pickled crucifers, mutagenic *N*-nitroso compounds may be generated.[113] Remember, however, that epidemiological data suggest that ingestion of cruciferous vegetables is associated with a decreased, rather than an increased, risk for carcinogenesis. The doses used for these mutagenicity and genotoxicity studies cannot easily be extrapolated for comparison to human dietary intake. Therefore, at normal dietary intake, the risk for genotoxicity may be less than the potential for anticarcinogenicity. If an individual were to be exposed to far higher than normal dietary doses, this relationship might change. Furthermore, epidemiological data are based entirely on intake of whole vegetables. Uptake, efficacy, and toxicity of purified compounds may be very different.

In rodent feeding studies aimed at evaluating anticarcinogenic effects, animals are typically fed 2.5 to 30% dry vegetable in their diets. In one study, when rats were fed >10% dry matter as Brussels sprouts, they exhibited growth depression and decreased food intake.[114] In one study, PEITC was given postinitiation and animals were seen to lose weight. It was hypothesized that calorie restriction, due to feed refusal, was the cause of the antitumorigenic effects.[69] Other studies have fed up to 30% dry matter as Brussels sprouts[37] without measurable decrease in food intake or growth compared with control animals. Others have chosen to pair-feed control animals, adjusting feed intake to the previous day's intake of the experimental animals to ensure that both groups take in the same number of calories.[102]

Cruciferous vegetables have been a part of the diet for many centuries. They are nutritious and, when eaten as part of the normal diet, are not associated with adverse health effects. However, safety is a matter of dose. Any compound exhibiting bioactivity can be expected to exert adverse, or toxic, effects at sufficiently high doses. There are two main syndromes affecting livestock who ingest abnormally high amounts of plants in the crucifer family. A hemolytic syndrome, *Brassica* anemia or kale poisoning, is due to the presence of SMCSO.[115] SMCSO is metabolized to dimethyl disulfide, which depletes erythrocyte glutathione, causing oxidative damage to erythrocyte membranes.[116] A similar syndrome is seen in animals consuming an excessive amount of garlic. The

oils from the cruciferous oilseeds rape and crambe are high in erucic acid, which causes cardiotoxicity. When the oil is pressed out for industrial use in plastics manufacture, the remaining meal may be fed to livestock as a protein source. Nonruminant livestock that are fed diets >10% rape, crambe, or other oilseed meals from the crucifer family exhibit feed refusal, growth depression, goiter, and liver and skeletal abnormalities. These adverse effects seen in livestock are considered to be due to glucosinolates and their hydrolysis products. The meal from industrial oilseeds is high in glucosinolates, often containing 3 to 7% glucosinolates by weight. In contrast, cruciferous vegetables contain 0.02 to 0.4% glucosinolate by weight, although levels in cruciferous vegetable seeds are typically tenfold higher than in the plant. Therefore, intake of cruciferous vegetable seeds as a significant percentage of the diet could produce adverse effects.

Cabbage is considered goitrogenic when consumed in excess. Goiter formation is dependent upon concomitant iodine deficiency, in which case thiocyanate ions inhibit iodine uptake sufficiently to cause iodine-deficient goiter.[22] In addition, a glucosinolate hydrolysis product found particularly in turnips exerts an antithyroid effect that is unaffected by iodine, but reversed by thyroxine. This product, a cyclic thiocyanate derivative of progoitrin termed goitrin, is thought to inhibit thyroxine synthesis. Again, these effects are associated with excess consumption rather than normal dietary intake.

Nitrile products from oilseeds have also been studied in detail because livestock have sometimes been exposed to excessive doses. Synthetic nitriles release cyanide upon hydrolysis and produce classic cyanide toxicosis. The cyano group on cruciferous nitriles is not released during metabolism. However, renal, hepatic, and pancreatic toxicities from nitriles derived from crucifers have been reported.[117,118] Fortunately, many nitriles are inherently unstable and do not persist in foods that have been cooked or even just stored for prolonged periods of time.

The evidence for carcinogenic and toxic effects of crucifers and their bioactive components needs to be put into the context of the new vegetable products that can be expected on the market in response to research on anticarcinogenesis of cruciferous vegetables. Any hydrolysis product developed as an anticarcinogenic agent for use as prophylactic therapy must pass through classic drug trials, both animal and clinical, to ensure safety prior to administration to patients. Extracts or other preparations sold as dietary supplements are not subject to such rigorous toxicologic evaluation. It rests on the shoulders of the producer to submit safety data to the FDA. These documents frequently consist of proposals that the substance be "generally recognized as safe" (GRAS), having historically been incorporated into the diet. Proposed dosage level is based on serving size. The statements may well be true but absorption characteristics of extracts or tablets may be very different from those of the whole vegetable. Furthermore, extract and tablet intake is not limited by bulk in the same manner as whole vegetables. Since it is not known how much an individual might ingest, believing that if a little is good, a lot may prove better, an upper dosage limit should be added to the package label to avoid misunderstandings of this nature.

IX. IMPACTING THE AMERICAN DIET

Functional foods such as broccoli and cabbage, which are already commonly consumed, can have a very real impact on the health of the nation if the consumer is knowledgeable about the product, its anticarcinogenic content, and the correct methods of preparation and storage. At present, broccoli varieties are not identified in the market, although varieties of many items, such as apples and potatoes, have been identified for a considerable time. Clearly, the potency of crucifer varieties can be expected to differ manyfold[20,21] (see Table 11.2). The consumer interested in obtaining the anticarcinogenic benefits from crucifers needs to be informed about the varieties on the market and which may offer the most healthful combinations of active compounds. Consumer information should also include information regarding the chemical makeup and methods for optimal preparation and storage to retain the anticarcinogenic health effects of crucifers.

The methods used in preparing vegetables can have profound effects upon the potency of the product as a chemopreventative agent. Although there are good indications that the hydrolysis products of glucosinolates are active anticarcinogens, the extent to which they are released from the parent glucosinolates may vary depending upon conditions of food preparation, altering the overall healthfulness of the products we eat. When glucosinolates isolated from Brussels sprouts were fed to rats, they had no measurable effect on clearance of the drug antipyrine unless they were first hydrolyzed by adding myrosinase. When the hydrolyzed products were fed to rats, antipyrine clearance was increased by 66%.[119] Information of this type has been used to suggest that glucosinolate breakdown products are the active molecules in upregulation of detoxification enzymes. This is important as one considers whether it is better to cook cruciferous vegetables or eat them raw to obtain their health benefits. Cooking the vegetables inactivates myrosinase and interferes with the hydrolytic conversion of the parent glucosinolates to their breakdown products. In the raw vegetables, myrosinase activity would be sustained and the conversion of glucosinolates to hydrolysis products could proceed. Whether or not significant hydrolysis could occur through such actions as autolysis during storage, or during the preparation of the vegetable for cooking, has not been fully assessed.[84] Undoubtedly, bringing the enzyme in contact with the stored glucosinolates would allow some immediate conversion of glucosinolates to breakdown products. The observation that feeding rats a diet supplemented with 20% cooked Brussels sprouts for 2 days[37] resulted in an upregulation of detoxification enzymes could be explained by partial hydrolysis of the glucosinolates during the preparative stages. In this study, the authors stored Brussels sprouts frozen until ready for use, then defrosted them before cooking, possibly permitting autolysis of glucosinolates during defrosting. This interpretation is further supported by the observation that fresh, freeze-dried, and cooked vegetables appear to have some ability to upregulate detoxification enzymes. Potency is enhanced when the crucifers are homogenized or chopped and allowed to stand prior to freezing.[83]

In the whole plant, when glucosinolates are hydrolyzed, either by crushing or chopping, both the isothiocyanate and the nitrile are formed.[10] One problem in considering the benefit of vegetable sources of isothiocyanates is that, while the isothiocyanate may be a potent anticarcinogen, the nitrile may be without activity.[120] For example, under acidic conditions, like those found in the stomach, sulforaphane nitrile is formed instead of sulforaphane from glucoraphanin.[98] Interestingly, when myrosinase is semipurified and added to a vegetable extract containing glucoraphanin, only sulforaphane is produced.[121] Clearly, there are differences between hydrolysis of glucoraphanin during digestion, in the whole vegetable *in situ*, and by semipurified myrosinase *in vitro*. Whether or not there are food preparation techniques that can easily promote the *in situ* formation of the isothiocyanate over the nitrile has yet to be determined.

One difficulty in assessing the potency of crucifers rests with differences in preparation of the plant materials. Most animal studies have been carried out using freeze-dried raw vegetables. While there are relatively few human studies, most have employed cooked vegetables. Feeding cooked cabbage (200 g/day) and Brussels sprouts (300 g/day) to humans caused an increase in detoxification enzymes,[32] and feeding cooked Brussels sprouts (300 g/day) decreased oxidative DNA damage.[122] Similarly, feeding cooked broccoli (500 g/day) was reported to alter detoxification enzymes.[123] Cooking typically destroys the hydrolyzing enzyme, myrosinase, but apparently did not destroy the ability of crucifers to upregulate detoxification enzymes in the human studies reported here. Alternatively, a study of the effect of fresh watercress consumption on metabolism of the tobacco carcinogen NNK reported upregulated detoxification.[67] Small amounts of information are beginning to accumulate that suggest that purified glucosinolates are bioactive when ingested. If hydrolysis of glucosinolate is not catalyzed by the plant myrosinase, due to its destruction by cooking, some other source of hydrolyzing activity may exist. Researchers have begun to question whether colonic bacteria could play a role in hydrolysis of glucosinolates.[124] At this time, there is no clear answer to this question.

In an attempt to introduce a high-potency broccoli product, Fahey and colleagues[121] have developed a product now sold as a dietary supplement, called broccoli sprouts. These are approximately

3-day sprouts from seeds of a high-glucoraphanin broccoli variety. The sprouts have glucoraphanin levels approximately tenfold greater than the mature plant on a wt/wt basis.[121] While Fahey and colleagues are to be lauded for applying their research, the consumer may require advice on use and preparation of the product to capitalize fully on the anticarcinogenetic effects. Would use of an acid salad dressing favor the formation of the inactive nitrile instead of bioactive sulforaphane from the parent glucosinolate? Should there be concern for the potential of sulforaphane toxicity? If individuals were to eat a large quantity of sprouts daily, they could be exposed to an amount of sulforaphane that has not been evaluated for toxicity.

Concern should also be raised that competitors may grow seedlings from less-potent varieties, or grow them for a longer period thereby losing the efficacy advantage over mature broccoli. By 10 days, the broccoli seedling has a similar glucoraphanin content to that of the parent plant. Most people would eat a larger portion of steamed broccoli than of fresh broccoli sprouts in a salad and may actually ingest less glucoraphanin, believing the sprouts to be more potent, than if they were to eat the mature plant. Three commercial varieties that are relatively high in glucoraphanin, the parent glucosinolate of sulforaphane, are Brigadier, Majestic, and Saga.[21,95] A group in Britain has developed a broccoli with unusually high levels of glucoraphanin by crossing a commercial variety with a wild variety.[125] However, this plant is not commercially available, and no high-potency varieties have yet appeared on the market.

Unfortunately, few reports include data regarding optimal levels of consumption of crucifers. While 200 to 300 g of cooked vegetable are effective in upregulating detoxification enzymes in humans,[32,122,123] the smallest effective serving size has not been reported. One meta-analysis of epidemiological data suggested that as few as 10 g/day could have a significant effect on reducing the risk for cancer.[8] Broccoli and cabbage rank in the top 10 vegetables purchased by U.S. households with broccoli sales suggesting that daily consumption is approximately 5 g/capita.[126] New broccoli products, including broccoflower (a cross between broccoli and cauliflower) and broccolini (a cross between broccoli and kale) are on the market, but glucosinolate levels in these varieties are not yet reported. Tablets containing broccoli and other cruciferous vegetables are becoming available in health food stores. One study reported that broccoli tablets cause induction of glutathione transferase activity in colonic mucosa of mice (1 g/kg body weight). When the same tablets were given to humans at high risk for colon carcinoma (3 g/day or <0.05 g/kg body weight), no effect was seen.[127] It should be noted that 3 g/day of a dehydrated preparation is equivalent to about 30 g/day fresh vegetable. While this dose is an order of magnitude less than doses shown to affect human detoxification enzyme activities, epidemiological data suggest it should be sufficient to have an effect on cancer incidence.

There is much work to be done to build an understanding of the interactions between varieties, glucosinolate content, environments for hydrolysis, content of hydrolysis products, preparation and storage techniques, and overall protection against cancer. It remains clear, however, that crucifers offer a valuable health benefit to the diet that may not be obtained through other functional foods. Only time remains before there are cruciferous cultivars commercially available that are nutritious, palatable, and sufficiently potent when integrated into the diet on a regular basis, to offer protection from the risks of cancer.

X. SUMMARY

A diet rich in cruciferous vegetables, such as broccoli and cabbage, is associated with a decreased risk for a number of cancers. Crucifers contain a family of secondary plant metabolites known as glucosinolates, that are fairly unique to these vegetables. Upon hydrolysis, glucosinolates yield a number of breakdown products, mostly isothiocyanates, that are biologically active. Several of these isothiocyanate derivatives, particularly phenylethyl isothiocyanate, sulforaphane, and indole-3-carbinol, have been shown to decrease tumor incidence when incorporated into the feed of animals exposed to chemical carcinogens. These same isothiocyanates have also been shown to increase or

upregulate several detoxification enzymes. For these reasons, the anticarcinogenic action of cruciferous vegetables is proposed to be due to isothiocyanate derivatives of glucosinolates, alone or in combination with other components, such as dithiolethione, the nitrile crambene, SMCSO, and several antioxidant vitamins, whose roles are not yet as clearly identified. Upregulation of detoxification enzymes by glucosinolate hydrolysis products is believed to enhance clearance of carcinogens resulting in decreased initiation of carcinogenesis. In addition to the proposed mechanism of induction of detoxification enzymes, it has been suggested that crucifers may act through inhibition of cytochrome P450-dependent bioactivation of carcinogens resulting in decreased initiation of cancer and, through cell cycle arrest and apoptosis, resulting in decreased progression of tumor growth. With cancer as the second leading cause of death in American society, the American public is interested in dietary means for lowering cancer risk. Researchers, in addressing this interest, are designing and bringing to market high-potency cruciferous vegetable products and purified glucosinolate hydrolysis products.

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12 Garlic: The Mystical Food in Health Promotion

John A. Milner

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I. INTRODUCTION

Garlic has been revered for its medicinal properties for centuries. This reverence has been propelled in recent years by the emergence of data revealing that in addition to antimicrobial properties it may also reduce the risk of heart disease and cancer.[1–4] The ability of garlic and related components to assist in maintaining immunocompetence[5,6] and possibly mental function[7] suggests its health implications could be extremely widespread.

Garlic is not simply a spice, herb, or vegetable, but a combination of all three. It along with onions, leeks and chives constitute the major *Allium* foods consumed by humans. The garlic bulb consists of several individual pieces, also known as bulblets or cloves, each weighting about 3 g. Actual garlic intakes are not known with certainty especially since it is not typically considered in dietary assessment surveys. Nevertheless, intakes are thought to vary from region to region and from individual to individual. Average intakes in the United States may be around 0.6 g/week or less,[8] while intakes in parts of China may be as much as 20 g/day.[9] While these exaggerated intakes in China can occur without ill consequences, not all individuals would be expected to consume garlic without some side effects. A spectrum of adverse allergic reactions can occur following contact with garlic, although the incidence is low.[10]

About 250 million pounds of garlic are produced annually in the United States. About 80 to 90% of this amount is produced in California. Significant production also occurs in Oregon, Nevada, and Arizona. Approximately 50 million pounds are sold yearly as fresh garlic. The remaining 200 million pounds are dehydrated to be used as flakes, salt, and in packaged foods. Garlic has continued to be one of the top-selling herbs in the United States. In 1998 garlic sales in the United States were approximately $230 million, about a 10% increase over 1997 sales.[11]

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II. THE CHEMISTRY OF GARLIC

The use of garlic typically centers on its unique flavor and odor characteristics. Unlike other foods, garlic is distinctive in that about 1% of its dry weight is sulfur.[12] Although garlic does not typically serve as a major source of essential dietary nutrients, it can add to a variety of different components (Table 12.1). Carbohydrates provide about 33% of its weight. Protein provides another 6.4% of the weight, much of which is relatively rich in arginine. Although many of the health benefits of garlic have been attributed to its sulfur components, its other constituents including selenium, oligosaccharides, and flavonoids may influence the magnitude of the response.[4,13,14]

The primary sulfur-containing constituents in garlic bulbs are γ-glutamyl-S-alk(en)yl-L-cysteines and *S*-alk(en)yl-L-cysteine sulfoxides. The content of *S*-alk(en)ylcysteine sulfoxide in garlic typically ranges between 0.53 to 1.3% of the fresh weight, with alliin (*S*-allylcysteine sulfoxide) the largest contributor.[15] Alliin concentrations can increase during storage as a result of the transformation of γ-glutamylcysteines. In addition to alliin, garlic bulbs contain small amounts of (+)-*S*-metyl-L-cysteine sulfoxide (methiin) and (+)-*S*-(*trans*-1-propenyl)-L-cysteine sulfoxide, *S*-(2-carboxypropyl)glutathione, γ-glutamyl-*S*-allyl-L-cysteine, γ-glutamyl-*S*-(*trans*-1-propenyl)-L-cysteine, and γ-glutamyl-*S*-allyl-mercapto-*L*-cysteine.[12]

The characteristic odor of garlic arises from allicin (thio-2-propene-1-sulfinic acid S-allyl ester) and oil-soluble sulfur compounds formed when the bulb is crushed or damaged. This membrane destruction yields a host of organosulfur degradation products as a result of the release of the vacuolar enzyme alliinase. This enzyme rapidly converts alliin to form the odiferous alkyl alkanethiosulfinates, including allicin. Since allicin is unstable, it further decomposes to sulfides, ajoene, and dithiins.[16–18] Tamaki and Sonoki[18] reported that strong garlic flavor and scent were linked to a higher content of volatile sulfur. Interestingly, heating garlic was associated with a reduction in allyl mercaptan, methyl mercaptan, and allyl methyl sulfide and a reduction in smell, possibly because of a reduction in alliinase activity.[18]

Little is known about the *in vivo* pharmokinetics of allyl sulfur compounds. While it is unlikely that allicin occurs to any extent once garlic is processed, if it does it would be rapidly transformed in liver to diallyl disulfide (DADS) and allyl mercaptan as suggested by studies by Egen-Schwind and colleagues.[19] Recently Teyssier and colleagues[20] concluded that diallyl disulfide might be

TABLE 12.1
Content of Selected Components in Edible Garlic

Component	Amount/100 g
Water g	58.6
Energy, kcal	149.0
Protein, g	6.4
Total lipid (fat), g	0.5
Carbohydrate, g	33.1
Fiber, total dietary, g	2.1
Calcium, mg	181.0
Magnesium, mg	25.0
Phosphorus, mg	153.0
Potassium, mg	401.0
Selenium, μg	14.200
Vitamin C, mg	31.200
Folate, μg	3.100

Source: USDA Nutrient Database for Standard Reference, Release 13 (November 1999).

reconverted to diallyl thiosulfinate (allicin) in tissues principally by oxidation arising from cytochrome P450 monooxygenases and to a limited extent by flavin-containing monooxygenases. Interestingly, their data suggest DADS is preferentially metabolized in human liver to allicin by CYP2E1. Since DADS can also cause the autocatalytic destruction of this enzyme, it is unclear how much allicin might be formed under physiological conditions. Flavin-containing monooxygenases in liver probably are responsible for the oxidization of *S*-allyl cysteine (SAC), among many other sulfur compounds.[21] P450 monooxygenases do not appear to be involved in SAC metabolism.

III. IMPLICATION IN HEALTH

Garlic and a host of its allyl sulfur compounds have been reported to possess a variety of health benefits. Notable among these are antimicrobial, anticarcinogenic, and protective benefits against cardiovascular disease. Table 12.2 contains a list of some of the most commonly used compounds that have been found to have benefits on a variety of biomarkers that reflect a reduction in risk. Although long-term intervention studies are lacking, a variety of laboratory-based and epidemiological studies suggest that key molecular targets involved in the risk of several diseases can be influenced by these compounds.

TABLE 12.2
Sulfur Compounds in Garlic with Potential Health Benefits Properties

E-Ajoene	Diallyl disulfide
Z-Ajoene	Diallyl sulfide
Allicin	Diallyl trisulfide
Allixin	*S*-Allyl cysteine
Allyl mercaptan	*S*-Allylmercaptocysteine
Allyl methyl sulfide	

A. Antimicrobial Effects

A host of plants are reported to act as antimicrobial agents. Those rich in tannins, terpenoids, alkaloids, flavonoids, and sulfur compounds have been found to be particularly effective. Historically, garlic extracts have been labeled as universal antibiotics.[22] Considerable evidence indicates that garlic extracts can inhibit a range of Gram-negative and Gram-positive bacteria, and serve as an antifungal agent.[23,24] In addition to allicin, various other sulfur compounds including diallyl sulfide (DAS), DADS, E-ajoene, Z-ajoene, E-4,5,9-trithiadeca-1,6-diene-9-oxide (E-10-devinylajoene, E-10-DA), and E-4,5,9-trithiadeca-1,7-diene-9-oxide (iso-E-10-devinylajoene, iso-E-10-DA) may contribute to the antimicrobial properties attributable to garlic.[1,25] Although differences in efficacy among these compounds exist, relatively small amounts are effective microbial growth deterrents. However, not all microorganisms are equally susceptible to the toxic effects of individual sulfur compounds.[26]

Helicobacter pylori colonization of the gastric mucosa is increasingly linked with gastritis. Likewise, emerging evidence connects gastritis with a greater propensity to develop gastric cancer. Studies by Cellini and colleagues[27] workers provide rather convincing evidence that aqueous garlic extracts (2 to 5 mg/ml) inhibit *H. pylori* proliferation. Reduced effectiveness occurred when the garlic was heated prior to extraction.[27] This depression in activity suggests that maximal activity may require that selected compounds be formed from alliin after the bulb is crushed. Since both DAS and DADS are recognized to elicited a dose dependent depression in *H. pylori* proliferation in culture,[28] a reduction in their formation may account for the loss of effectiveness arising from

heating. Variation in the efficacy of various garlic preparations is to be expected since the minimum inhibitory concentration (MIC) is observed to range from 10 to 17.5 mg/ml for raw garlic extracts and three commercially available garlic tablets.[29] The unique sensitivity of microorganisms to specific sulfur compounds from garlic is amply demonstrated in the studies by Sivam and colleagues[26] in which 40 μg of thiosulfinate/ml was the MIC for *H. pylori* but did not influence *Staphylococcus aureus* growth.

Allium foods including garlic are also effective in suppressing fungal growth.[24] Allicin has been reported to be protective against *Candida albicans* and a host of other strains. These organisms were extremely sensitive to garlic extracts, some to a greater degree than to nystatin, a known effective antibiotic.[23] Ajoene is also noted for its antimycotic activity both *in vitro* and *in vivo*. A fungal infection of the skin, known commonly as ringworm and medically as tinea corporis, can also be influenced by sulfur compounds found in garlic. Ledezma and colleagues[30] found that treatment with ajoene (0.4% gel) was as effective as terbinafine (1% cream) in healing tinea corporis and tinea cruris in 60 soldiers with dermatophytosis. Since ajoene can be prepared easily from garlic, it may be particularly useful as a public health strategy particularly in developing countries

The main antimicrobial effect of sulfur compounds in garlic may reflect chemical reactions with selected thiol groups of various enzymes and/or a change in the overall redox state of the organism. As discussed below, changes in thiol status has been suggested as one possible mechanism by which garlic and related sulfur compounds might suppress tumor proliferation.[31] Both alliin and allicin are recognized to possess antioxidant properties in a Fenton oxygen-radical-generating system [H_2O_2–Fe(II)].[32] DADS, but not diallyl sulfide, dipropyl sulfide, or dipropyl disulfide, has been found to inhibit liver microsomal lipid peroxidation induced by NADPH, ascorbate, and doxorubicin.[33] Water-soluble SAC has also been shown to possess antioxidant activity.[34,35]

B. Cancer

Scientists, legislators, and consumers are becoming increasingly aware that several foods may contribute to health, including a reduction in cancer risk.[36,37] Although major limitations exist in defining the precise role that garlic has in the cancer process, the likelihood of its significance is underscored by both epidemiological and laboratory investigations. Laboratory-based studies with model cancers provide some of the most compelling evidence that garlic and its related sulfur components can suppress cancer risk and alter the biological behavior of tumors.

1. Nitrosamine Formation

Experimentally, garlic and its associated components have been found to suppress the incidence of breast, colon, skin, uterine, esophagus, and lung cancers.[13,39–44] This protection may arise from several mechanisms, including blockage of *N*-nitroso compounds (NOCs) formation, suppression of the bioactivation of several carcinogens, enhanced DNA repair, reduced cell proliferation, and/or induction of apoptosis. It is possible, and quite probable, that several of these cellular events are modified simultaneously.

Considerable evidence points to the ability of garlic to suppress the formation of NOCs.[45–49] NOCs are potent carcinogens in a variety of biological systems and also presumably in humans.[50] Human exposure to these suspect carcinogens occurs through the ingestion or inhalation of preformed NOCs or by the ingestion of precursors that are combined endogenously.[51] The reduction in NOCs may actually be secondary to an increase in the formation of nitrosothiols. Williams[52] proposed that several sulfur compounds could foster the formation of nitrosothiols thereby reducing the quantity of nitrite available for NOC formation. Studies by Dion[25] revealed that not all allyl sulfur compounds are equally effective in stopping the formation of NOCs. The ability of SAC and its non-allyl analogue *S*-propyl cysteine to retard NOC formation, but not DADS, dipropyl disulfide, and diallyl sulfide

reveals the critical role that the cysteine residue has in inhibition.[25] Since the content of allyl sulfur can vary among preparations, it is certainly probable that not all garlic sources are equal in the protection they provide against NOC formation. Some of the protection against NOC exposure may also relate to antimicrobial properties associated with garlic and some of its components.[25]

Some of the most compelling evidence that garlic depresses nitrosamine formation in humans comes from studies by Mei and colleagues.[53] In their studies providing 5 g of garlic per day completely blocked the enhanced urinary excretion of nitrosoproline that occurred as a result of ingesting supplemental nitrate and proline. The significance of this observation comes from the predictive value that nitrosoproline has for the synthesis of potential carcinogenic nitrosamines.[54] Evidence that the effect of garlic occurs with nitrosamines other than those excreted in urine comes from data from Lin and colleagues.[55] Their studies provided evidence that garlic was effective in blocking liver DNA adducts resulting from the feeding of NOC precursors.

The anticancer benefits attributed to garlic are also associated with the ability of its allyl sulfur compounds to suppress carcinogen bioactivation. Evidence from a variety of sources reveals that garlic is effective in blocking DNA aklylation, a primary step in nitrosamine carcinogenesis.[55–57] Consistent with this reduction in bioactivation, Dion and colleagues[47] found that both water-soluble SAC and lipid-soluble DADS were effective in retarding the mutagenicity of NMOR in *Salmonella typhimurium* TA100. A block in mutagenicity following aqueous garlic extract exposure has also been noted following treatment with ionizing radiation, peroxides, adriamycin, and *N*-methyl-*N'*-nitro-nitrosoguanidine.[58]

A block in nitrosamine bioactivation may reflect changes in several enzymes. However, substantial evidence points to the involvement of cytochrome P450 2E1 (CYP2E1).[5,59] An autocatalytic destruction of CYP2E1 may account for some of the chemoprotective effects of diallyl sulfide, and possibly other allyl sulfur compounds.[60] Variation in the content and overall activity of P450 2E1 may be an important variable in the degree of protection provided by garlic and associated allyl sulfur components.

2. Other Carcinogens

Garlic and several of its allyl sulfur compounds can also effectively block the bioactivation and carcinogenicity of non-NOCs (Table 12.3). This protection, which involves a diverse array of compounds and several target tissue sites, suggests either multiple mechanisms of action or a widespread biological effect. Since metabolic activation is required for many of the carcinogens used in these studies there is likelihood that phase I and II enzymes are involved. Interestingly, little, if any, change in cytochrome P450 1A1, 1A2, 2B1, or 3A4 activities have been observed following treatment with garlic or related sulfur compounds.[61–63] Thus, other enzymes involved in the bioactivation or removal of carcinogenic metabolites might be involved in the observed protection. Singh and colleagues[64] provided evidence that the efficacy of various organosulfides to suppress benzo(a)pyrene tumorigenesis was correlated with their ability to suppress NAD(P)H:quinone oxidoreductase, an enzyme involved with the removal of quinones associated with this carcinogen. Changes in bioactivation resulting from a block in cyclooxygenase and lipoxygenase may also partially account for the reduction in tumors following treatment with some carcinogens.[65–69] Changes in glutathione concentration and the activity of specific glutathione-*S*-transferase (GST), both factors involved in phase II detoxification, may be important in the protection provided by garlic. Feeding garlic powder to rats has been found to increase the activity of GST in both liver and mammary tissue.[61,70,71] However, not all GST isozymes may be influenced equally. Hu and colleagues[72] provided evidence that the induction of glutathione (GSH) *S*-transferase pi (mGSTP1–1) may be particularly important in the anticarcinogenic properties associated with garlic and allyl sulfur components.

Cancer progression is probably also highly dependent on epigenetic changes. Two extensively examined mechanisms for epigenetic gene-regulation are (1) patterns of DNA methylation and (2)

TABLE 12.3
Carcinogens Influenced by Garlic and/or Associated Allyl Sulfur Compounds[a]

Compound	Site	Host
1,2-Dimethylhydrazine	Colon	Rat
3-Methylcholanthrene	Cervix	Mouse
4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone	Nasal	Rat
7,12 Dimethylbenz(a)anthracene	Mammary	Rat
	Skin	Mouse
	Forestomach	Hamster
	Buccal Pouch	Hamster
Aflatoxin B1	Liver	Toad
	Liver	Rat
Azoxymethane	Colon	Rat
Benzo(a)pyrene	Forestomach	Mouse
	Lung	Mouse
	Skin	Mouse
	Bone Marrow	Mouse.
Methylnitronitrosoguanidine	Gastric	Rat
N-Methyl-*N*-nitrosourea	Mammary	Rat
N-Nitrosodiethylamine	Colon	Rat
	Nasal	Rat
	Liver	Rat
	Nasal	Rat
	Skin	Mouse
N-Nitrosomethylbenzylamine	Esophagus	Rat
Vinyl carbamate	Skin	Mouse

[a] The overall response to garlic and/or specific allyl sulfur components depends on the quantity provided and the amount of carcinogen administered.

histone acetylations/deacetylations. Several studies indicate that DNA hypermethylation is an important mechanism for inactivation of key regulatory genes such as E-cadherin, pi-class GST, the tumor suppressors cyclin-dependent kinases (CDKN2) and the phosphatase gene (PTEN), and insulin-like growth factor (IGF-II) targeted histone acetylation/deacetylation results in remodeling of chromatin structure and correlates with activation/repression of transcription (e.g., IGFBP-2 and p21). Both DNA methylation and histone acetylation can be modified by garlic and or related allyl sulfur compounds. Studies by Ludeke and colleagues[73] found that DAS inhibited the formation of O^6-methyldeoxyguanosine in esophagus (26%), nasal mucosa (51%), trachea (68%), and lung (78%) that arose from treatment with *N*-nitrosomethylbenzylamine. Likewise, other studies reveal DADS, SAC, and deodorized garlic are effective in retarding the DNA methylation caused by MNU.[55,74] Lea and colleagues[75] reported that at least part of the ability of DADS to induce differentiation in DS19 mouse erythroleukemic cells might relate to its ability to increase histone acetylation. Diallyl disulfide caused a marked increased in the acetylation of H4 and H3 histones in DS19 and K562 human leukemic cells. Consistent with other studies the disulfide was more effective that the monosulfide. Similar results were also obtained with rat hepatoma and human breast cancer cells. Allyl mercaptan was a more potent inhibitor of histone deacetylase than diallyl disulfide. Interestingly, DADS has been also been reported to inhibit the growth of H-ras oncogene transformed tumors in nude mice.[76] This inhibition correlated with the inhibition of p21H-ras membrane association in the tumor tissue.

Garlic has also been found to reduce the incidence of tumors resulting from the treatment with methylnitrosurea (MNU), a known direct-acting carcinogen.[55] Water-soluble SAC (57 μmol/kg diet)

and lipid-soluble DADS cause a comparable reduction in MNU induced O^6-methylguanine adducts bound to mammary cell DNA.[74] Most recently, SAC was not found to provide protection against MNU-induced mammary tumors.[77] The reason for this discrepancy is unknown but may relate to the quantity of lipid in the diet or the quantity of carcinogen provided. If there truly are effects of DADS and SAC on MNU carcinogenesis, the mechanism(s) by which it brings about this effect are unclear. It is possible that garlic could influence mammary gland terminal end bud formation and/or a change in rates of DNA repair.

Rarely has a comparison of water- and oil-soluble compounds been conducted within the same study. Nevertheless, available evidence suggests that major differences in efficacy among extracts are not of paramount importance.[42,74,78–81] Although subtle differences among garlic preparations are likely to occur, quantity rather than source appears to be a key factor influencing the degree of protection.[78] Differences that do occur between preparations likely relate to the content and effectiveness of individual sulfur compounds. The number of sulfur atoms present in the molecule seems to influence the degree of protection with diallyl trisulfide > diallyl disulfide > diallyl sulfide.[82–84] Likewise, the presence of the allyl group generally enhances protection over that provided by the propyl moiety.[72,83]

3. Diet as a Modifier

The influence of garlic on the cancer process cannot be considered in isolation since several dietary components can markedly influence its overall impact. Among the factors recognized to influence the response to garlic are total fat, selenium, methionine, and vitamin A.[42,,85,86] Amagase and colleagues[42] and Ip and colleagues[85] reported that selenium supplied either as a component of the diet or as a constituent of the garlic supplement, respectively, enhanced the protection against 7,12-dimethylbenz(a)anthracene (DMBA) mammary carcinogenesis over that provided by garlic alone. Suppression in carcinogen bioactivation, as indicated by a reduction in DNA adducts, may partially account for this combined benefit of garlic and selenium.[86] However, both selenium and allyl sulfur compounds are recognized to alter cell proliferation and induce apoptosis.[83,87,88]

Dietary fatty acid supply can also dramatically influence the bioactivation of DMBA to metabolites capable of binding to rat mammary cell DNA. A significant portion of the enhancement in mammary DNA adducts caused by increasing dietary corn oil consumption can be attributed to linoleic acid intake.[86] Although exaggerated oleic acid consumption also increases DMBA-induced DNA adducts, it is far less effective in promoting adducts compared with linoleic acid. The ability of selected fatty acids to alter DMBA bioactivation may provide clues to a plausible mechanism by which garlic and its allyl sulfur compounds might retard chemically induced tumors. As indicated previously, it does not appear that changes in cytochrome P450 enzymes can account for the protection provided by supplemental garlic. Smith and colleagues[89] reported that prostaglandin H synthase could metabolize the bay region diol of benzo(a)pyrene to electrophilic diol epoxides that were capable of binding to DNA. Most recently, our laboratory has reported that DMBA bioactivation appears to depend on cyclooxygenase activity.[86] Ali[90] provided evidence that garlic can inhibit cyclooxygenase. Studies by McGrath and Milner[69] provided evidence that both water and lipid soluble allyl sulfur compounds could retard the ability of cyclooxygenase to bioactivate DMBA. A close examination of the rate of formation of adducts in *in vitro* DMBA bioactivation studies suggests the involvement of possibly another enzyme. A logical enzyme to consider is lipoxygenase since it is reported to bioactivate several carcinogens[67,68] including benz(a)pyrene,[65,66] a carcinogen similar to DMBA. McGrath and Milner[69] reported that lipoxygenase could bioactivate DMBA. Interestingly, the activation caused by lipoxygenase was about ten times greater than that caused by cyclooxygenase. While limited, there are some data indicating that garlic and associated sulfur components can inhibit lipoxygenase activity.[91] Finally, evidence for the involvement of lipoxygenase in the bioactivation of DMBA comes from data from Song and Milner.[44] They reported that feeding the known

lipoxygenase inhibitor, nordihydroguaiaretic acid (NDGA), was accompanied by a marked reduction in DMBA-induced DNA adducts in rat mammary tissue. Collectively, these studies pose interesting questions about the role of both cyclooxygenase and lipoxygenase in not only in forming prostaglandins, and therefore modulating tumor cell proliferation and immunocompetence, but also in their involvement in the bioactivation of carcinogens. Clearly, additional attention is needed to clarify what role, if any, these enzymes have in determining the biological response to dietary garlic or its allyl sulfur components.

C. Heart Disease

Garlic may have a role in the genesis and progression of cardiovascular disease through a variety of biological effects including a decrease in total and low-density lipoprotein (LDL) cholesterol, increase in high-density lipoprotein (HDL) cholesterol, reduction of serum triglyceride and fibrinogen concentrations, lowering of arterial blood pressure, and/or an inhibition of platelet aggregation. Several studies have attempted to clarify the exact role that garlic has on serum cholesterol, LDL, HDL, and triglycerides since these might be signals of protection.[3,92–97] Although some studies have reported that garlic reduces LDL concentrations,[98,99] others have not.[100] Unraveling the true response is complicated by the use of various quantities of garlic, different preparations and standardizations, and variation in the duration of treatment. Nevertheless, many do provide evidence that garlic can decrease cholesterol and triglyceride concentrations in some, but probably not all, patients. Cholesterol reductions in the range of 7 to 15% tend to be the norm. McCrindle and colleagues[101] did not detect a significant effect of garlic in children with hypercholesterolemia. Whether this relates to the quantity of garlic (300 mg/day), the duration (8 weeks), or to the particular children is unclear. Likewise, the lack of a response in the double-blind randomized study by Berthold and colleagues[102] indicates that not all people will likely benefit and/or that the preparation (steam-distilled garlic oil) or the amount provided (5 mg twice daily) may influence the response. LDL oxidation is increasing being recognized as a contributor to the initiation and progression of atherosclerosis.[103–105] This oxidation occurs when LDL is exposed to free radicals released by surrounding cells such as smooth muscles cells, or monocytes/macrophages.[104,105] Munday and colleagues[106] found a reduced susceptibility of LDL particles to Cu^{+2}-mediated oxidation from subjects given 2.4 g of aged garlic extract (AGE) daily for 7 days. Interestingly, a similar response was not observed when subjects were given 6 g of raw garlic. Byrne and colleagues[100] did not find that 900 mg of Kwai for 6 months had an impact on LDL susceptibility to oxidation. It is unclear if the discrepancies in the literature about garlic and LDL oxidation relate to the subjects examined or to the preparations used. Clearly, additional studies are needed to resolve this issue.

Aortic stiffening is also an important risk factor in cardiovascular morbidity and mortality. This stiffness coincides with a high systolic blood pressure and augmented pulse pressure. Diet in addition to age, gender, hormonal state, and genetic factors probably influence aortic stiffening. Increasing evidence suggests garlic may be a dietary component with the ability to alter blood pressure and cause relaxation in arterial walls. Recently, garlic was found in the Goldblatt model for hypertension to exert a sustained depression in arterial blood pressure.[107] Garlic treatment has also been found to lead to a dose-dependent vasorelaxation in both endothelium-intact and mechanically endothelium-disrupted pulmonary arterial rings.[108] This vasorelaxation was diminished by the administration of NG-nitro-L-arginine methyl ester, a nitric oxide synthase inhibitor. The inducible nitric oxide synthase (iNOS) is recognized to occur in human atherosclerotic lesions and is thought to promote the formation of peroxynitrites. Allicin and ajoene have been reported to cause a dose-dependent inhibition of the iNOS system in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages.[109] This inhibition was correlated with a reduction in iNOS protein, as well as in its mRNA. Thus, changes in NO concentrations may be key in the observed response to garlic and associated sulfur components.

Acute coronary syndromes can occur when an unstable atherosclerotic plaque erodes or ruptures, thereby exposing the highly thrombogenic material inside the plaque to the circulating blood.[110] This exposure triggers a rapid formation of a thrombus that occludes the artery. Efendy and colleagues[111] found that feeding a deodorized garlic preparation (Kyolic) reduced the fatty streak development and vessel wall cholesterol accumulation in cholesterol-fed rabbits. Similarly, garlic consumption for 48 weeks in a randomized trail was found to reduce arteriosclerotic plaque volumes in both the carotid and femoral arteries by 5 to 18%.[112]

Aggregates of activated platelets also likely have a pivotal role in coronary syndromes. Garlic and some of its organosulfur components have been found to be potent inhibitors of platelet aggregation *in vitro*.[113–115] Heating of garlic by boiling retards its ability to inhibit platelet aggregation.[115] Unfortunately, few studies have documented that garlic can inhibit platelet aggregation *in vivo*. However, Steiner and Lin[116] provided evidence that consumption of aged garlic extract reduced epinephrine- and collagen-induced platelet aggregation, although it failed to influence adenosine diphosphate (ADP)-induced aggregation. Their studies also provided evidence that platelet adhesion to fibrinogen could be suppressed by consumption of this garlic supplement. Overall, the ability of garlic to reduce hyperlipidemia, hypertension, sterol synthesis, and thrombus formation makes it a strong candidate for lowering the risk of heart disease and stroke. Nevertheless, considerably variability is observed. Additional studies are needed to clarify who might benefit most from added garlic as a protective measure against cardiovascular disease.

D. Garlic and Immunocompetence

Diet is increasingly recognized to play an important role in the development and functionality of immunocompetent cells. Several dietary components including allyl sulfur compounds may have physiologically important immunomodulatory effects.[117] DAS treatment of BALB/c mice has been reported to block the suppression of the antibody response caused by *N*-nitrosodimethylamine to T-cell-dependent antigens, and the lymphoproliferative response to T-cell and the B-cell mitogens.[5] Likewise, other compounds may also be effective immunomodulators. Romano and colleagues[118] reported that ajoene significantly retarded the proliferation induced in human lymphocytes by the mitogens phytohemagglutinin (PHA), phorbol myristate acetate (PMA), and anti-CD3, and the capping formation induced in B lymphocytes by anti-IgM antibodies. Ajoene was also found to inhibit partially the lypopolysaccharide-induced production of tumor necrosis factor (TNF) and to decrease the phagocytic activity of thioglycolate-elicited mouse peritoneal macrophages for IgG-opsonized, human erythrocytes.[118] However, the effects are not limited to sulfur compounds since a protein fraction isolated from aged garlic extract was found to enhance cytotoxicity of human peripheral blood lymphocytes (PBL) against both natural-killer (NIK)-sensitive K562 and NK-resistant M14 cell lines.[6] The mechanism by which sulfur or nonsulfur components of garlic influence immunocompetence remains to be determined.

IV. SUMMARY AND CONCLUSIONS

Garlic is a plant within in the genus *Allium* with significant physiological attributes for promoting health. Although it is possible that other allium foods possess similar health attributes, few comparative studies have been undertaken. Its relatively few side effects should not detract from its use, except possibly its lingering odor. However, odor does not appear to be a necessary prerequisite for most of the benefits since water-soluble SAC generally gives comparable benefits to those compounds associated with smell. It is probably that garlic and its associated water- and lipid-allyl sulfur compounds influence several key molecular targets in disease prevention. While most can savor the culinary experiences identified with garlic, some individuals because of their gene profile and/or environmental exposures may be particularly responsive to more exaggerated intakes.

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13 Antioxidant Vitamin and Phytochemical Content of Fresh and Processed Pepper Fruit (*Capsicum annuum*)

Luke R. Howard

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I. INTRODUCTION

Capsicum species are a New World crop belonging to the Solanacae family. Chiles have been cultivated for thousands of years, and they are one of the oldest domesticated crops. Most cultivars grown in the United States belong to the species *C. annuum* and are typically classified according to fruit shape, flavor, and culinary uses. In addition to *C. annuum* species, *C. frutescens* (tabasco) and *C. chinense* (habanero) are commonly cultivated and used for culinary and medicinal purposes. The classification and varieties of peppers grown in the United States,[1,2] and the production, technology, chemistry, and quality of *Capsicum* spp. have been reviewed extensively.[3–7]

Capsicum spp. exhibit great genetic diversity in terms of color, size, shape, and chemical composition. Researchers have recently recognized that *Capsicum* fruit also vary greatly in their content of antioxidant vitamins and phytochemicals. This information may be important for human health and nutrition as consumers incorporate more peppers into their diets. The goal of this chapter is to survey the antioxidant vitamin and phytochemical content of different *Capsicum* species, types, and cultivars, and to determine the effects of postharvest handling and processing on the levels of these important phytonutrients.

0-8493-8734-5/01/$0.00+$.50
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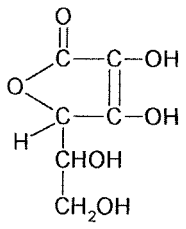
II. FRUITS AND VEGETABLES AND DISEASE PREVENTION

Epidemiological studies indicate that antioxidants present in fruits and vegetables, including β-carotene and vitamins C and E, may be important in prevention of numerous degenerative conditions, including various types of cancer, cardiovascular disease, stroke, atherosclerosis, and cataracts.[8-10] Oxidative damage catalyzed by reactive oxygen species (ROS) has been implicated in over 100 degenerative conditions.[11] ROS cause damage to cellular membranes, proteins, and DNA, which increases the susceptibility of cells to chronic diseases. Oxidative damage in the body is exacerbated when the balance of ROS exceeds the amount of endogenous antioxidants. The human body has several enzymatic and nonenzymatic defense systems to regulate ROS *in vivo*, but these defense mechanisms are thought to deteriorate with aging. Consumption of fruits and vegetables that are rich in antioxidant nutrients may afford additional protection against ROS-mediated disorders. Scientists have recently recognized that fruits and vegetables are not only a good source of antioxidant vitamins, but also an excellent source of other essential dietary phytochemicals that can retard the risk of degenerative diseases.[12] The potential health effects of phytochemicals are associated with numerous mechanisms, including prevention of oxidant formation, scavenging of activated oxidants, reduction of reactive intermediates, induction of repair systems, and promotion of apoptosis.[13]

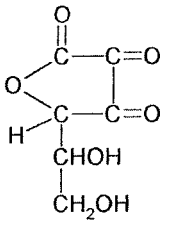
III. ASCORBIC ACID

Capsicum fruit have long been recognized as an excellent source of ascorbic acid, which is a required nutrient for humans. Svent-Gyorgyi isolated ascorbic acid from paprika fruit in the early 1930s, and subsequently identified the compound in 1933.[14] Ascorbic acid has strong reducing properties due to its enediol structure, which is conjugated with the carbonyl group in a lactone ring[15] (Figure 13.1). In the presence of oxygen, ascorbic acid is degraded to dehydroascorbic acid (DHA), which still retains vitamin C activity. However, upon further oxidation, the lactone ring of DHA is destroyed, resulting in formation of 2,3-diketogulonic acid and loss of vitamin activity. Ascorbic acid is required for collagen formation and prevention of scurvy. Researchers have postulated a role of ascorbic acid in the prevention of degenerative conditions, including cancer, heart disease, cataracts, and stimulation of the immune system.[16] Prevention of chronic diseases may be attributed to the ascorbate function as an aqueous reducing agent. Ascorbate can reduce superoxide, hydroxyl, and other ROS, which may be present in both intracelluar and extracellular matrices. Ascorbate within cells participates as an electron donor as part of the interaction between iron and ferritin. Extracellularly, ascorbate may act in concert with tocopherols in lipid membranes to quench ROS and prevent lipid peroxidation. Thus, ascorbate may help prevent the oxidation of low-density lipoprotein (LDL), which is thought to be a major initiating step in the process of atherosclerosis. The role of ascorbate in cancer prevention may be attributed to its ability to block the formation of *N*-nitrosamines and nitrosamides, compounds that induce cancer in experimental animals, and possibly humans.[16]

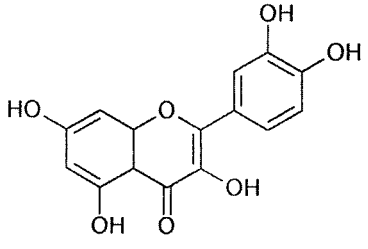
The ascorbic acid content of pepper cultivars from several species is shown in Table 13.1.[17-21] All the peppers referenced are an excellent source of ascorbic acid, with most of the cultivars contributing over 100% of the RDA (60 mg/100 g). The only pepper cultivars that fail to meet the recommended daily allowance (RDA) for ascorbic acid are the "chile" type cv. NuMex RNaky at the green stage, and the "tabasco" type cv. tabasco at the green stage. The ascorbic acid content of most of the pepper types increases during ripening, with much higher levels found in mature peppers at the final stage of ripening. Higher levels of ascorbic acid observed during ripening may be related to light intensity and greater levels of glucose, the precursor of ascorbic acid.[22] Since total and reducing sugars increase during pepper fruit ripening, the elevated ascorbic acid levels in mature fruit may reflect greater synthesis due to the higher levels of sugar precursors.[21] In addition to maturation, variation in ascorbic acid content between pepper types and cultivars may be attributed to differences in genetics, fertilization practices,



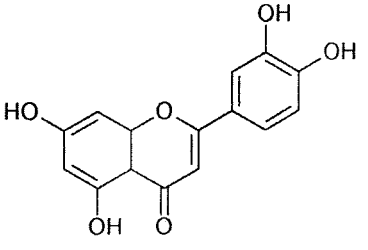
L-ascorbic acid



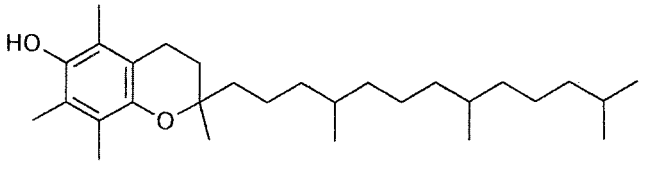
L-dehydroascorbic acid



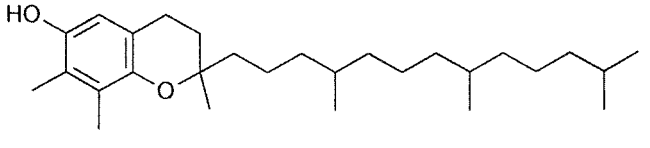
Quercetin



Luteolin



α-tocopherol



γ-tocopherol

FIGURE 13.1 Ascorbic acid, flavonoids, and tocopherols in *Capsicum* fruit.

and environmental growing conditions. The effects of fertilization on ascorbic acid content of peppers have been studied. Ascorbic acid content of pepper fruits increased with increasing levels of phosphorus up to 48 kg/ha, at varying levels of nitrogen (0 to 100 kg/ha),[23] and applications including combinations of organic matter + nitrogen + phosphorus increased ascorbic acid content of *Capsicum* fruit.[24] Application of bioregulators may also affect ascorbic acid content of peppers. The ascorbic acid and citric acid contents of "bell" peppers increased with gibberellic acid treatment.[25]

TABLE 13.1
Ascorbic Acid Content of Fresh *Capsicum* Fruit

Species	Type	Cultivar	Maturity	mg/100 g Fresh Weight	% RDA[a]	Ref.
annuum	Ancho	San Luis Ancho	Green	168	280	17
	Bell	Dove	White	77	128	18
		Dove	Light Orange	103	172	18
		Ivory	White	89	148	18
		Ivory	Light Yellow	110	183	18
		Blue Jay	Purple	95	158	18
		Blue Jay	Orange	123	205	18
		Lilac	Purple	67	112	18
		Lilac	Orange	104	173	18
		Valencia	Green	119	198	18
		Valencia	Orange	73	122	18
		Oriole	Green	91	152	18
		Oriole	Orange	86	143	18
		Black Bird	Green	66	110	18
		Black Bird	Black	62	103	18
		Chocolate Beauty	Green	62	103	18
		Chocolate Beauty	Brown	100	167	18
		Cardinal	Green	102	170	18
		Cardinal	Red	124	207	18
		King Arthur	Green	84	140	18
		King Arthur	Red	87	145	18
		Var. 862R	Green	88	147	18
		Var. 862R	Red	98	163	18
		Red Bell G	Green	95	158	18
		Red Bell G	Red	96	160	18
		Red Bell C	Green	72	120	18
		Red Bell C	Red	107	178	18
		Klondike Bell	Green	112	187	18
		Klondike Bell	Yellow	109	182	18
		Canary	Green	112	187	18
		Canary	Yellow	108	180	18
		Orobelle	Green	162	270	18
		Orobelle	Yellow	95	158	18
		Golden Bell	Green	106	177	18
		Golden Bell	Yellow	90	150	18
		Tam Bel-2	Green	109	182	19
		Tam Bel-2	Red	148	247	19
		Grande Rio-66	Green	98	163	19
		Grande Rio-66	Red	149	248	19
		Yellow Bell-47	Green	114	190	20
		Yellow Bell-47	Orange	135	225	20
	Cascabella	Peto Cascabella	Yellow	172	287	20
		Peto Cascabella	Red	202	337	20
	Cayenne	Mesilla	Green	63	105	20
		Mesilla	Red	102	170	20
	Chile	New Mexico-6	Green	141	235	19
		New Mexico-6	Red	205	342	19
		New Mexico-6	Green	130	217	17
		Tam Mild Chile	Green	155	258	19

TABLE 13.1 (CONTINUED)
Ascorbic Acid Content of Fresh *Capsicum* Fruit

		Tam Mild Chile	Red	233	388	19
		Green Chile	Green	122	203	17
		Sandia	Green	71	118	21
		Sandia	Breaker	220	367	21
		Sandia	Red	239	398	21
		New Mexico 6-4	Green	40	67	21
		New Mexico 6-4	Breaker	122	203	21
		New Mexico 6-4	Red	155	258	21
		NuMex RNaky	Green	28	47	21
		NuMex RNaky	Breaker	145	242	21
		NuMex RNaky	Red	164	273	21
		B-18	Green	15	25	21
		B-18	Breaker	91	152	21
		B-18	Red	186	310	21
	Jalapeno	Jalapeno-M	Green	173	288	19
		Jalapeno-M	Red	179	298	19
		Tam Veracruz	Green	101	168	19
		Tam Veracruz	Red	144	240	19
		Tam Veracruz	Green	72	120	17
		Tam Mild	Green	66	110	17
		Mitla	Green	49	82	17
		Jaloro	Yellow	131	218	17
		Sweet Jalapeno	Green	54	90	17
	Serrano	Hidalgo	Green	141	235	19
		Hidalgo	Red	263	438	19
	Yellow Wax	Hungarian Yellow	Yellow	114	190	17
		Long Hot Yellow	Yellow	114	190	17
		Gold Spike	Yellow	115	192	17
		Inferno	Yellow	92	153	20
		Inferno	Red	138	230	20
		Rio Grande Gold	Red	243	405	19
		Sante Fe Grande	Red	187	312	19
chinense	Habanero	Red Savina	Red	192	320	20
		Francisca	Orange	203	338	20
frutescens	Tabasco	McIhenny Tabasco	Green	15	25	20
		McIlhenny Tabasco	Red	75	125	20

[a] = RDA, male = 60 mg/100 g, female = 60 mg/100 g.

Effects of Postharvest Handling and Processing on Ascorbic Acid Content

The ascorbic acid content of peppers is influenced by postharvest handling, packaging, and processing. Fresh peppers are sensitive to chilling injury, so they should be stored at 8 to 12°C, at relative humidities of 90 to 95%. Under optimum conditions, peppers may be stored for 2 to 3 weeks after harvest. However, the nutritional quality of peppers may change after harvest, handling, and transportation en route to various markets, especially under abusive handling conditions. The ascorbic acid content of sweet bell peppers from wholesale and retail markets and simulated consumer storage was reported to be similar, although a wide range of ascorbic acid was apparent among individual market samples.[26] Thus, it appears that the average concentration of ascorbic acid does not change appreciably from wholesale marketing to consumption. The ascorbic acid content

of bell peppers was influenced by storage temperature, but not by packaging in perforated films.[27] Ascorbic acid levels declined 10% after 4 days storage at 10°C, whereas a 25% loss occurred after 4 days storage at 20°C. In contrast, the ascorbic acid content of bell peppers was unaffected by storage temperature (2 and 8°C), varying levels of carbon dioxide (5, 10, 20%), or storage time (6, 9, and 12 days).[28] In another study, the ascorbic acid content of bell peppers increased with storage at 13°C, and with subsequent ripening at 20°C.[29]

Ascorbic acid content of fresh peppers may also be affected by postharvest chlorinated water treatments. Green bell peppers dipped in 50, 100, 150, and 200 μg/ml hypochlorite lost 6, 9, 10, and 18% of their initial total ascorbic acid concentrations, respectively.[30] It was recommended that chlorine concentrations of 50 to 100 μg/ml during a 20-min contact time could be used to control microbial spoilage without affecting overall quality of bell peppers.

Although the effects of modified-atmosphere storage on ascorbic acid retention in whole fresh bell peppers are conflicting, minimally processed peppers appear to benefit from modified atmosphere storage. Precut jalapeno peppers stored in modified-atmosphere packages (MAP; 5% O_2 and 4% CO_2) retained 85% of their ascorbic acid after 15 days storage at 13°C, while air-stored peppers retained only 56%.[31] The MAP treatment also retarded the conversion of L-ascorbic to dehydroascorbic acid. Similar results were reported for sweet blanched bell peppers stored in reduced-oxygen atmospheres.[32] Ascorbic acid levels were better retained in storage atmospheres of 2% O_2 and 4% O_2, than samples stored in air. Conversion of ascorbic acid to dehydroascorbic acid was also retarded under reduced-oxygen storage.

Due to its water solubility, ascorbic acid is readily leached from pepper fruit during water blanching and pasteurization in salt-acid brines. Jalapeno peppers that were blanched prior to pasteurization lost 75% of their ascorbic acid,[19] while a 40% loss of ascorbic acid occurred during water blanching of green bell peppers, and a 15% loss occurred during steam blanching.[33] Ascorbic acid content of unblanched "yellow banana" peppers declined substantially during pasteurization and storage, with only 10% remaining after 124 days.[34] Calcium chloride brine treatment did not affect ascorbic acid retention in pasteurized yellow banana peppers. In contrast, initial ascorbic acid levels were retained in jalapeno peppers after blanching and pasteurization.[35] Blanching may also affect ascorbic acid retention in frozen peppers. Unblanched "padron" peppers lost 97% of their ascorbic acid within 1 month of freezing, while blanching resulted in a 28% loss, followed by an additional 10% loss after 12 months frozen storage.[36] In another study, ascorbic acid losses of ten pepper cultivars that were blanched and stored for 12 months at –12°C were 63%, while unblanched cultivars lost 71%.[37] Differences in ascorbic acid losses in these studies may be attributed to differences in pepper genetics, brine composition, blanching method, and pasteurization time and temperature.

Ascorbic acid content of dehydrated peppers is influenced by blanch and drying methods. "Paprika" fruit lost 63% of its ascorbic acid content when naturally dried, while losses of 4 to 54% were observed when freshly harvested and overripe fruit were dried using a forced-air method.[38] Other processing parameters may also influence ascorbic acid retention. A 40% loss of ascorbic acid in paprika powder was noted after centrifugation prior to drying, and a 73% loss occurred in carmelized paprika.[39] In another study, drying time and temperature did not affect the ascorbic acid content of dehydrated green bell peppers, but after 8 weeks of storage, blanched peppers dried for 8 h at 60°C contained less ascorbic acid than unblanched peppers.[40] Unblanched peppers dried for 12 h at 49°C contained more ascorbic acid than blanched peppers.

IV. FLAVONOIDS

Pepper fruit are particularly rich in flavonoids, a large class of compounds ubiquitous in plants, that exhibit antioxidant activity, depending on the number and location of hydroxyl groups present.[41] In addition to antioxidant function, flavonoids are reported to possess numerous biological, pharmacological, and medicinal properties, including vasodilatory, anticarcinogenic,

immune-stimulating, antiallergenic, antiviral, and estrogenic effects, as well as inhibition of various enymes involved in carcinogenesis.[42] In addition, many epidemiological studies indicate an inverse association between the intake of flavonols and flavones and the risk of coronary heart disease,[43–45] stroke,[46] and lung cancer.[47,48]

The flavonoid content of different pepper types and cultivars is shown in Table 13.2. Peppers contain both quercetin (a flavonol) and luteolin (a flavone). Quercetin has a hydroxyl group at C-3 in the aromatic ring, while luteolin does not (see Figure 13.1). The structural differences are important since the presence of a hydroxyl group at C-3 is reported to result in greater free radical–scavenging efficiency.[41] In plant cells, flavonoids occur as glycosides, with sugars bound typically at the C-3 position. Flavonoids are typically quantified in the aglycone form after acid

TABLE 13.2
Flavonoid Content of Fresh *Capsicum* Fruit

						Total	
				Quercetin	Luteolin	Flavonoids	
Species	Type	Cultivar	Maturity	mg/kg Fresh Weight			Ref.
annuum	Ancho	San Luis Ancho	Green	276	34	310	20
	Bell	Yellow Bell	Green	22	11	33	17
		Yellow Bell	Orange	13	9	22	17
		Tam B-2	Green	44	9	53	50
		Romanian Sweet	Yellow	219	26	245	50
		YB 244	Yellow	81	10	91	50
		YB 126	Yellow	112	15	127	50
	Cascabella	Peto Cascabella	Yellow	42	16	58	17
		Peto Cascabella	Red	24	6	30	17
		Tam Cascabella	Yellow	67	30	97	50
	Cayenne	Mesilla	Green	25	17	42	17
		Mesilla	Red	11	6	17	17
	Chile	New Mexico-6	Green	126	51	177	20
		Green Chile	Green	210	52	262	20
	Jalapeno	Mitla	Green	40	14	54	20
		Tam Mild	Green	18	10	28	20
		Jaloro	Yellow	151	38	189	20
		Sweet Jalapeno	Green	45	6	51	20
		TAES Jaloro	Yellow	52	18	70	50
	Serrano	Hidalgo	Green	160	41	201	20
	Yellow Wax	Hungarian Yellow	Yellow	784	68	852	20
		Long Hot Yellow	Yellow	447	104	551	20
		Gold Spike	Yellow	288	37	325	20
		Inferno	Yellow	68	17	85	17
		Inferno	Red	65	17	82	17
		Short Sweet Yellow	Yellow	88	18	106	50
		Long Sweet Yellow	Yellow	56	9	65	50
		Short Hot Yellow	Yellow	62	14	76	50
		Long Hot Yellow	Yellow	78	15	93	50
		TAES Hot Yellow	Yellow	79	17	96	50
		Sweet Banana	Yellow	43	6	49	50
chinense	Habanero	Francisca	Orange	5	1	6	17
		Red Savina	Red	1	ND	1	17
frutescens	Tabasco	McIlhenny Tabasco	Green	2	44	46	17
		McIlhenny Tabasco	Red	1	36	37	17

hydrolysis. The flavonoids in yellow wax peppers were identified as quercetin rhamnoside, luteolin glucoside, and luteolin 7-*o*-apiosylglucoside.[49] Flavonoid levels vary greatly among pepper types and cultivars with total levels ranging from 1 to 852 mg/kg. The exceptionally high flavonoid levels reported by Lee and colleagues[17] may be due to differences in genetics and environmental conditions in which the peppers were grown. Environmental stress during plant growth has been shown to stimulate the phenylpropanoid pathway and production of various phenolic compounds.

The flavonoid content in fresh *Capsicum* fruit is shown in Table 13.2.[50] Typically, peppers that contain high levels of quercetin also contain high levels of luteolin. *Capsicum* fruit appear to be unique in that they contain quercetin and luteolin, both of which exhibit excellent free radical-scavenging properties due to their structural similarities.[41] Interestingly, *C. annuum* cultivars contain higher levels of flavonoids than *C. chinense* cultivars. Low levels of flavonoids in the pungent *C. chinense* peppers may indicate diversion of phenolic precursors from flavonoid to capsaicinoid synthesis. An exception is the *C. frutescens* cv. tabasco, which contains much higher levels of luteolin than the other *Capsicum* species and cultivars. It appears that fruit from different *Capsicum* species vary greatly in their genetic capacity for synthesizing specific flavonoids. Plant breeders and molecular biologists may take advantage of this genetic variability to increase the flavonoid content of *Capsicum* fruit.

Increasing luteolin levels in pepper fruit may be important for prevention of coronary heart disease. A luteolin-rich artichoke extract was recently shown to protect LDL from oxidation *in vitro*, which may be due to its antioxidant function or ability to sequester pro-oxidant metal ions.[51] Additionally, luteolin does not complex with copper ions to produce oxidative damage to DNA, which contrasts with the pro-oxidant effect observed for quercetin.[52]

Total flavonoid content of pepper cultivars generally declines as fruit ripens and changes color. Exceptions include the cayenne cv. Mesilla, in which the flavonoid content increased during maturation, and the "long yellow" cv. Inferno, and tabasco cv. Tabasco, in which no change in flavonoid content occurred during ripening. The loss of flavonoids observed during ripening of most cultivars is consistent with reported flavonoid losses that occur during maturation of *C. frutescens* fruit.[53] Flavonoid losses during ripening may reflect metabolic conversion of flavonoids to secondary phenolic compounds.[54] The oxidoreductase enzymes polyphenol oxidase[55] and peroxidase[56,57] may play a role in degradation of flavonols during maturation and senescence.

Postharvest Handling and Effect of Processing on Flavonoid Content

Little information is available on the effects of postharvest handling and processing on flavonoid content of pepper fruit. The effect of pasteurization and storage on flavonoid content in yellow banana peppers has been studied.[34] Quercetin and luteolin contents declined 40 to 45% during 4 months storage, while calcium chloride brine treatment did not affect flavonoid retention. Apparently, flavonoids are leached into the salt-acid brine during pasteurization and storage. Future studies should focus on methods to stabilize flavonoids during postharvest handling and processing.

V. TOCOPHEROLS

Capsicum fruit, especially in the dried form, are an excellent source of tocopherols. Vitamin E compounds including tocopherols and tocotrienols are well recognized for their effective inhibition of lipid oxidation in foods and biological systems.[58] The tocopherols are polyisoprenoid derivatives, which have a saturated C16 side chain (phytol), centers of asymmetry at the 2, 4′, and 8′ positions, and variable methyl substitution at R1, R2, and R3[15] (see Figure 13.1). The antioxidant activity of tocopherols is due to their ability to donate their hydrogen ions to lipid free radicals, thereby neutralizing the radical and forming the tocopheroxy radical. Tocopherols have been shown to be effective scavengers of peroxyl and superoxide radicals in lipid systems. Epidemiological and short-term intervention studies suggest that vitamin E may reduce the risk of coronary heart disease,

some cancers, cataracts, and diabetes, and slow the progression of neurological diseases. The health effects of vitamin E may be related to numerous mechanisms, including protection of cells from oxidative damage; protection of LDL from oxidation; enhancement of the immune system; reduction of oxidative damage of specialized tissues such as the eye lens, nerve tissue, blood vessels, and cartilage; reduction of cholesterol synthesis by inhibition of the enzyme HMG-Co A reductase; and enhancement of the antioxidant status of the digesta.[59]

The α-tocopherol content of pepper types and cultivars is shown in Table 13.3.[60,61] γ-Tocopherol is found in pepper seeds, whereas α-tocopherol is found in pericarp tissue. Dried paprika and "New Mexico" type peppers used in the spice industry are a fair source of γ-tocopherol, and an excellent source of α-tocopherol. New Mexico type cultivars contain higher levels of γ-tocopherol in seeds than paprika cultivars.[21] At the red succulent stage, the γ-tocopherol content of seeds from four New Mexican pepper cultivars ranged from 35.2 to 47.5 mg/100 g.[21] These levels of γ-tocopherol would provide 3.5 to 4.8% of the RDA for males and 4.4 to 5.9% of the RDA for females, per 1-g serving.

TABLE 13.3
Tocopherol Content of Fresh *Capsicum* Fruit

				α-Tocopherol[a]			
					%RDA[b]		
Species	Type	Cultivar	Maturity	Pericarp (mg/100 g DW)	M	F	Ref.
annuum	Paprika	Vandel	Green	16	160	200	60
		Vandel	Red	28	280	350	60
		Gamba	Green	17	170	210	60
		Gamba	Red	33	330	410	60
		Mild	Green	26	260	330	60
		Mild	Green-red	46	460	580	60
		Mild	Red	65	650	810	60
		Mild	Red-dried	68	680	850	60
		SZ-20	Ripe	47	470	590	61
		Mihalyteleki	Ripe	33	330	410	61
		SZ-80	Ripe	38	380	480	61
		F-03	Ripe	61	610	760	61
		SZ-178	Ripe	57	570	710	61
		Km-622[c]	Green	17	170	210	39
		Km-622[c]	Breaker-1	48	480	606	39
		Km-622[c]	Breaker-2	85	850	1,060	39
		Km-622[c]	Faint red	92	920	1,150	39
		Km-622[c]	Deep red	109	1,090	1,360	39
		Km-622[d]	Green	34	340	430	39
		Km-622[d]	Breaker-1	35	350	440	39
		Km-622[d]	Breaker-2	43	430	540	39
		Km-622[d]	Faint red	48	480	606	39
		Km-622[d]	Deep red	115	1,150	1,440	39
		Mihalyteleki	Green	43	430	540	38
		Mihalyteleki	Breaker-1	39	390	490	38
		Mihalyteleki	Breaker-2	42	420	530	38
		Mihalyteleki	Faint red	38	380	480	38
		Mihalyteleki	Deep red	40	400	500	38
		K-50	Deep red	180	1,800	2,250	38
		Km-622	Deep red	192	1,920	2,400	38

continued

TABLE 13.3 (CONTINUED)
Tocopherol Content of Fresh *Capsicum* Fruit

	K-801	Deep red	161	1,610	2,010	38
	Semi.-Determ. 7/92	Deep red	297	2,970	3,710	38
	SZ-80	Deep red	139	1,390	1,740	38
	K-V2	Deep red	205	2,050	2,560	38
	K-90	Deep red	265	2,650	3,310	38
	Strain-100	Deep red	141	1,410	1,760	38
	Mihalyteleki	Deep red	133	1,330	1,660	38
	SZ-20	Deep red	152	1,520	1,900	38
	Bibor	Deep red	95	950	1,190	38
	Napfeny	Deep red	90	900	1,130	38
	Negral	Deep red	87	870	1,090	38
New Mexico	Sandia	Green	5	50	60	21
	Sandia	Breaker	9	90	110	21
	Sandia	Red	17	170	210	21
	Sandia	Red-partially dry	10	100	130	21
	Sandia	Red-dried	19	190	240	21
	New Mexico 6-4	Green	6	60	80	21
	New Mexico 6-4	Breaker	6	60	80	21
	New Mexico 6-4	Red succulent	16	160	200	21
	New Mexico 6-4	Red-partially dry	12	120	150	21
	New Mexico 6-4	Red-dried	7	70	90	21
	NuMex R-Naky	Green	3	30	40	21
	NuMex R-Naky	Breaker	8	80	100	21
	NuMex R-Naky	Red succulent	13	130	160	21
	NuMex R-Naky	Red-partially dry	11	110	140	21
	NuMex R-Naky	Red-dried	21	210	260	21
	B-18	Green	2	20	30	21
	B-18	Breaker	8	80	100	21
	B-18	Red succulent	24	240	300	21
	B-18	Red-partially dry	15	150	190	21
	B-18	Red-dried	31	310	390	21

[a] α-Tocopherol = 1.0 mg αTE.
[b] RDA, male = 10 mg αTE, female = 8 mg αTE.
[c] Fruit harvested and analyzed in 1994.
[d] Fruit harvested and analyzed in 1995.

The α-tocopherol content of pericarp tissue in both paprika and New Mexico cultivars is exceptionally high, but the paprika cultivars are a better source of α-tocopherol. Per 100 g serving paprika cultivars provide 160 to 2970% of the RDA for males and 200 to 3710% of the RDA for females, while the New Mexico type cultivars provide 20 to 310% of the RDA for males and 30 to 390% of the RDA for females. Although small amounts of dried *Capsicum* powders are typically used for food preparation, their exceptionally high levels of tocopherols may be an important source of vitamin E in the human diet. A 1-g serving of dried paprika would provide 16 to 297% of the RDA for vitamin E for males and 20 to 371% of the RDA for females, while a similar serving of dried New Mexican peppers would provide 2 to 31% of the RDA for males and 3 to 39% of the RDA for females. Thus, dried peppers may be a significant source for vitamin E as people incorporate greater amounts of ethnic foods containing dried peppers into their diets.

The α- and γ-tocopherol content of pepper fruit is influenced by maturity. γ-Tocopherol content in seeds generally increases until the red succulent stage and then declines, while α-tocopherol content in pericarp tissue increases from the mature green to red fully dry stages.[21,39,60] The α-tocopherol content in pericarp tissue is dependent on lipid content, which varies according to ripening stage and variety.[60] A high correlation exists between oil content and α-tocopherol content in dry matter. The percentage of oil and α-tocopherol content is highest in red dried paprika fruit with 80% dry matter.

Effect of Processing on Tocopherol Content of Peppers

Color retention of dried paprika powder may be related to levels of γ-tocopherol in seeds,[63] but conflicting results are reported. Several studies[39,62] showed that color retention of paprika was improved with the addition of seeds, while other studies[61,63] found that the color stability of paprika was unaffected by addition of seeds. Conflicting results obtained between the studies may be related to varying levels of α-tocopherol in the peppers studied. Tocopherol content of dried paprika powder may also be influenced by cultivar, maturity, and drying method.[38] Tocopherol retention was lower in naturally dried samples than in forced-air-dried paprika. The α-tocopherol content increased during natural drying, reaching a maximum concentration when the dry matter of the fruit was between 53 and 68%, while a decrease in tocopherol content was observed with fully dry fruits having a dry matter content of 89%. For forced-air-dried fruit, utilization of fresh fruit as the starting material resulted in substantial losses of α-tocopherol. The best retention of α-tocopherol was obtained by drying overripe fruit having 53 to 68% dry matter. Two cultivars evaluated (Km-622 and V-2) lost 12.4 and 41.2% of α-tocopherol, respectively, when their overripe fruits were dried by the forced-air method. Thus, genetic variation should be taken into account when investigating the processing quality of new paprika cultivars. Tocopherol content is affected by additional processing parameters, including predrying centrifugation and carmelization during drying.[39] The α-tocopherol content of "paprika fruit" was highest in carmelized samples, indicating that carmelization of sugar afforded protection against tocopherol degradation during drying. The α-tocopherol content of centrifuged paprika was lower than values from noncentrifuged samples, indicating that it was removed from paprika fruit during the centrifugation step.

VI. CAROTENOIDS

Varying composition and concentration of carotenoids in *Capsicum* are responsible for diversely colored fruit. Common carotenoids in *Capsicum* fruits are shown in Figure 13.2. *Capsicum* spp. have been selectively bred to obtain fruit with various colors including white, green, yellow, orange, brown, black, and purple. The ketocarotenoids capsanthin and capsorubin contribute to red color, while α- and β-carotene, zeaxanthin, lutein, and β-cryptoxanthin are responsible for yellow-orange color. The carotenoids and additional pigments responsible for exotic colored brown and purple fruit have not been characterized.

The pepper carotenoids α- and β-carotene and β-cryptoxanthin contribute to provitamin A activity. However, pepper fruit are also a good source of oxygenated carotenoids, which vary considerably in composition and concentration due to differences in genetics and fruit maturation.[39,64] Oxygenated carotenoids, which do not possess provitamin A activity, have been shown to be effective free radical scavengers,[65] and may be important for prevention of advanced age-related macular degeneration and cataracts.[66] In addition to antioxidant activity, carotenoids may play a role in cancer chemoprevention through their ability to act as antimutagens,[67] enhance cell-to-cell communication,[68] act as anti-inflammatory and antitumor agents, and induce detoxification of enzyme systems.[69] Carotenoids present in pepper fruit may also be important in retarding changes associated with aging. Incorporation of a capsanthin-rich red bell pepper extract in the diet of senescence-accelerated mice resulted in amelioration of learning impairment.[70]

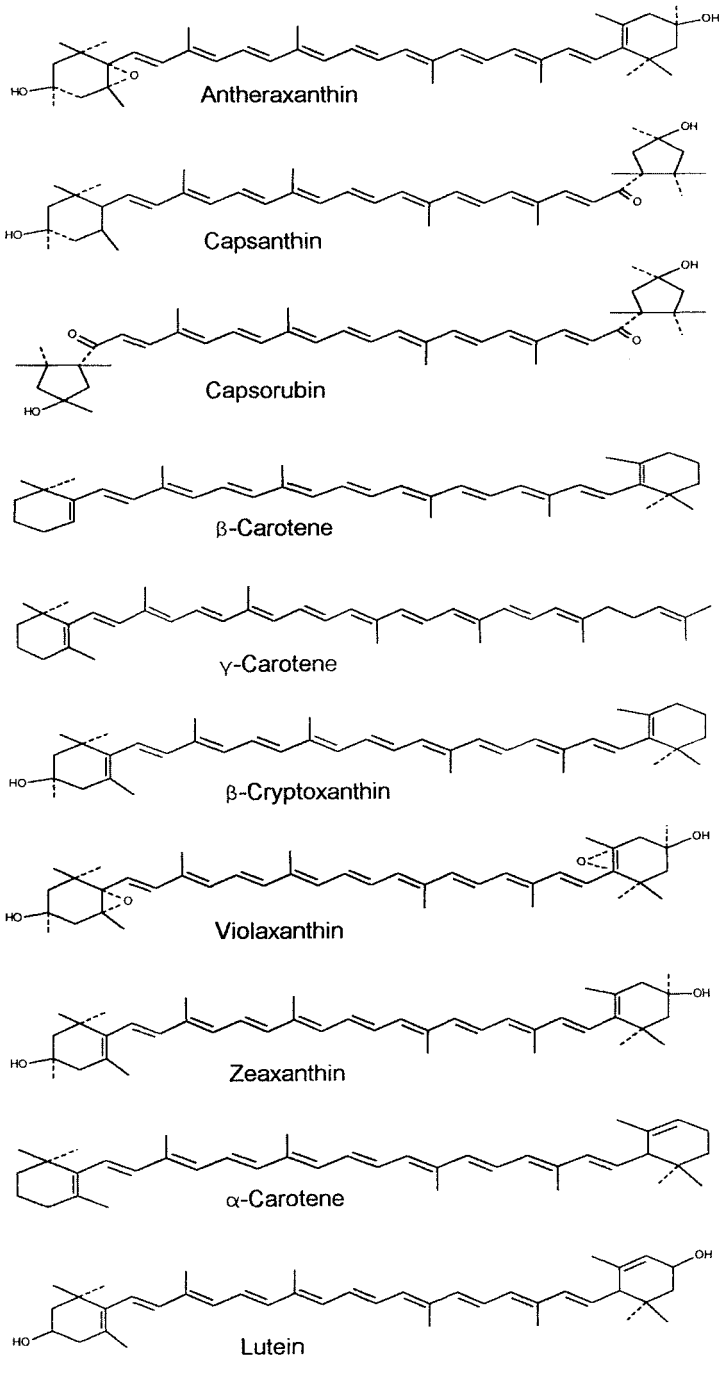


FIGURE 13.2 Major carotenoids in *Capsicum* fruit.

The provitamin A content (α- and β-carotene and β-cryptoxanthin) of fresh pepper cultivars is shown in Table 13.4.[71] Provitamin A values range from 2 to 502 retinal equivalents (RE)/100 g. The wide range of provitamin A carotenoids in the cultivars referenced is not surprising, since the cultivars vary greatly in visual color. Cultivars that terminally ripen at the green, light-yellow, or

TABLE 13.4
Provitamin A Content of Fresh *Capsicum* Fruit

Species	Type	Cultivar	Maturity	RE/100 g Fresh Weight	% RDA[a] M	% RDA[a] F	Ref.
annuum	Bell	Dove	White	16	1.6	2.0	18
		Dove	Light orange	29	2.9	3.6	18
		Ivory	White	14	1.4	1.8	18
		Ivory	Light yellow	46	4.6	5.8	18
		Blue Jay	Purple	22	2.2	2.8	18
		Blue Jay	Orange	59	5.9	7.4	18
		Lilac	Purple	17	1.7	2.1	18
		Lilac	Orange	86	8.6	10.8	18
		Valencia	Green	25	2.5	3.1	18
		Valencia	Orange	26	2.6	3.3	18
		Oriole	Green	37	3.7	4.6	18
		Oriole	Orange	99	9.9	12.4	18
		Black Bird	Green	32	3.2	4.0	18
		Black Bird	Black	41	4.1	5.1	18
		Chocolate Beauty	Green	38	3.8	4.8	18
		Chocolate Beauty	Brown	108	10.8	13.5	18
		Cardinal	Green	33	3.3	4.1	18
		Cardinal	Red	110	11.0	13.8	18
		King Arthur	Green	33	3.3	4.1	18
		King Arthur	Red	127	12.7	15.9	18
		Var. 862R	Green	38	3.8	4.8	18
		Var. 862R	Red	119	11.9	14.9	18
		Red Bell G	Green	44	4.4	5.5	18
		Red Bell G	Red	80	8.0	10.0	18
		Red Bell C	Green	35	3.5	4.4	18
		Red Bell C	Red	52	5.2	6.5	18
		Klondike Bell	Green	40	4.0	5.0	18
		Klondike Bell	Yellow	31	3.1	3.9	18
		Canary	Green	31	3.1	3.9	18
		Canary	Yellow	31	3.1	3.9	18
		Orobelle	Green	49	4.9	6.1	18
		Orobelle	Yellow	36	3.6	4.5	18
		Golden Bell	Green	37	3.7	4.6	18
		Golden Bell	Yellow	32	3.2	4.0	18
		Tam Bel 2	Green	33	3.3	4.1	18
		Tam Bel-2	Red	64	6.4	8.0	18
		Grande Rio-66	Green	81	8.1	10.1	18
		Grande Rio-66	Red	253	25.3	31.6	18
		Yellow Bell	Green	31	3.1	3.9	20
		Yellow Bell	Yellow	257	25.7	32.1	20
	Caribe	Caloro	Green	2	0.2	0.3	71
	Cascabella	Peto Cascabella	Yellow	4	0.4	0.5	20
		Peto Cascabella	Red	137	13.7	17.1	20
	Cayenne	Mesilla	Green	42	4.2	5.3	20
		Mesilla	Red	214	21.4	26.8	20
	Chile	New Mexico-6	Green	79	7.0	8.8	19
		New Mexico-6	Red	259	25.9	32.4	

TABLE 13.4 (CONTINUED)
Provitamin A Content of Fresh *Capsicum* Fruit

		Tam Mild Chile	Green	69	6.9	8.6	19
		Tam Mild Chile	Red	470	47.0	58.8	19
	Jalapeno	Jalapeno-M	Green	56	5.6	7.0	19
		Jalapeno-M	Red	168	16.8	21.0	19
		Tam Veracruz	Green	64	6.4	8.0	19
		Tam Veracruz	Red	252	25.2	31.5	19
		Tam Mild Jalapeno	Green	44	4.4	5.5	19
		Tam Mild Jalapeno	Red	83	8.3	10.4	19
	Poblano	Ancho	Green	111	11.1	13.9	71
	Serrano	TAES Hidalgo	Green	141	14.1	17.6	19
		TAES Hidalgo	Red	263	26.3	32.8	19
		Tampiqueno 74	Green	87	8.7	10.9	71
	Yellow Wax	Rio Grande Gold	Yellow	502	50.2	62.8	19
		Sante Fe Gold	Yellow	27	2.7	3.4	19
		Inferno	Yellow	4	0.4	0.5	20
		Inferno	Red	104	10.4	13.0	20
	Verde	Anaheim	Green	30	3.0	3.8	71
chinense	Habanero	Francisca	Orange	ND	ND	ND	20
		Red Savina	Red	191	19.1	23.9	20
frutescens	Tabasco	McIlhenny Tabasco	Green	29	2.9	3.6	20
		McIlhenny Tabasco	Red	336	33.6	42.0	20

[a]RDA, male = 1000 μg/RE, female = 800 μg/RE.

yellow stages typically have less provitamin A activity due to reduced genetic capacity to synthesize α- and β-carotene and β-cryptoxanthin.[64,72,73] Many studies have demonstrated that the β-, ε-series carotenoids in *Capsicum* fruit, including lutein, decline during ripening, whereas α- and β-carotene increase, and other pigments are formed *de novo*: β-cryptoxanthin, capsanthin, and zeaxanthin.[39,64,73] However, some exceptions are notable. The bell cv. Yellow Bell contains 257 RE/100 g and the yellow wax cv. Rio Grande Gold contains 502 RE/100 g. These yellow cultivars apparently contain the genetic capacity to synthesize provitamin A carotenoids.

Pepper fruit that ripen to orange and red stages contain high levels of provitamin A carotenoids, contributing 3 to 47% of the RDA (1000 RE/100 g) for males and 3 to 59% of the RDA (800 RE/100 g) for females. Pepper cultivars that contain >25% of the RDA of provitamin A for males and females include bell cv. Grande Rio 66 (red), bell cv. Yellow Bell (yellow), chile cv. Tam Mild Chile (red), chile cv. New Mexico-6 (red), jalapeno cv. Tam Veracruz (red), serrano cv. TAES Hidalgo (red), yellow wax cv. Rio Grande Gold (yellow), and tabasco cv. McIlhenny tabasco (red).

Carotenoids in paprika cultivars have been studied extensively, since they are used in the form of powders and oleoresins as spices and food colorants. Dried paprika products are exceptionally rich in carotenoids, including the ketocarotenoids capsanthin and capsorubin, which occur only in red *Capsicum* fruit, and contribute to the red color and quality of paprika oleoresin and powder. Numerous studies have documented the composition and concentration of carotenoids in dried paprika products that vary greatly in color.[63,74–78]

Efforts have been made to increase the carotenoid content of dried paprika through plant-breeding programs. The total carotenoid content of *C. annuum* breeding lines ranged from 390 to 16,600 μg/g.[76] Most of the red pigments were esterified, and diesters were present at higher concentrations than monoesters. An important observation was that a narrow range of variation existed among the ratios of carotenoids present in the breeding lines. This indicates that the levels of synthesis and accumulation of various carotenoid pigments are controlled genetically by common regulatory genes. Plant breeders may take advantage of this genetic trait in breeding

and developing new paprika cultivars with elevated levels of carotenoids, including the red pigments capsanthin and capsorubin, which affect the color and quality of paprika oleoresin and powder. One breeding line 4126, was identified,[76] which contained 240 mg of total carotenoids/100 g fresh weight, of which 20 mg was β-carotene. This level of β-carotene is comparable to levels found in carrots, but the total carotenoid content of this paprika breeding line is sixfold higher than levels found in carrots. Thus, breeding new pepper cultivars for elevated total carotenoid content appears to be feasible, and may be beneficial for improving human health and nutrition.

Dried paprika is an excellent source of vitamin A. A 1-g serving of freshly ground paprika provides 71 to 128% and 89 to 160% of the RDA for males and females, respectively.[38] Thus, dried paprika is an excellent source of various carotenoid pigments, which may be important for prevention of chronic diseases.

The carotenoid content of fresh and dried paprika fruit is also affected by the ripening stage. The β-carotene content of the fresh paprika fruit cv. Mihalyteleki increased from 419 μg/g dry matter at the green stage to 468 μg/g at the deep red stage, while the β-carotene content of dried fruit increased from 610 μg/g at the green stage to 652 μg/g at the deep red stage.[38]

Effects of Postharvest Handling and Processing on Carotenoid Content

The carotenoid content of peppers is influenced by postharvest handling, storage time, and temperature. Green bell peppers obtained from a roadside market on the day of harvest had much greater levels of α- and β-carotene than peppers purchased from a supermarket, which incurred 7 to 14 days of storage and transportation.[79] Storage temperature may also influence carotenoid retention. Yolo Wonder green bell peppers stored for 7 and 14 days at 7.2° and 21°C retained 94 and 78% of carotene, respectively.[80]

Major carotenoid losses may occur when peppers are physically wounded during minimal processing. A 32% loss of β-carotene and 48% loss of α-carotene occurred when jalapeno peppers were sliced into rings and stored in perforated packages for 12 days at 40°C, and an additional 3 days at 13°C.[31] Modified atmosphere storage (5% O_2 and 4% CO_2) protected against carotenoid degradation, with losses of 13 and 8% for β- and α-carotene, respectively, when pepper rings were stored under similar conditions as air-stored peppers. The beneficial effect of MAP storage on carotenoid retention may be due to inhibition of the enzymes peroxidase and lipoxygenase, which have been shown to cause destruction of β-carotene[81,82] or reduced chemical oxidation, due to the low oxygen levels inside the packages.

Carotenoids are better retained than ascorbic acid during pasteurization in salt-acid brines due to their hydrophobic properties, which prevents leaching into the brine solution. Pasteurization resulted in 13% reduction in β-carotene in mature green jalapenos and 31% reduction in mature red jalapenos,[19] while a 30% decrease in β-carotene occurred in pasteurized red bell peppers.[83] Loss of provitamin A in mature green jalapenos during pasteurization was 17%, while mature red jalapenos lost 33%.[19]

Carotenoid loss is recognized as the major cause of color degradation in dried red pepper products. Loss of the provitamin A carotenoids, β-carotene, and β-cryptoxanthin is associated with color loss during paprika processing. The red pigments capsorubin and capsanthin are more stable during drying and milling than the yellow pigments β-carotene and β-cryptoxanthin.[73] Esterified carotenoids show a greater degree of stability during processing than unesterified or free carotenoids. Thus, the ketocarotenoids capsanthin and capsorubin, which occur predominately in the diester form, are more stable than zeaxanthin, which occurs in the free, monoesterfied, or diesterified forms, β-cryptoxanthin, which occurs in only the free and monoesterified forms, and β-carotene, which occurs only in the free form. Greater susceptibility of free and monoesterified carotenoids to drying and milling results in provitamin A activity losses of 67 and 81% in the paprika varieties Agridulce and Bola, respectively.[73]

In addition to carotenoid composition and maturity, additional factors influencing carotenoid stability in dried red peppers include levels of natural antioxidants, water activity, drying temperature and technique, particle size, package atmosphere, and storage atmosphere and temperature. Dehydration of fresh ripe paprika fruit by forced-air drying resulted in 8% loss of β-carotene, while overripe fruit dried using the same technique lost 53 to 55%.[38] Forced-air drying resulted in greater retention of β-carotene in dried paprika than naturally dried fruit. Higher drying temperatures are also detrimental to color stability of dried paprika.

The concentrations of natural antioxidants present in paprika have been shown to affect color and carotenoid stability. β-Carotene concentration in paprika fruit declined slightly during the first 2 months of storage, and then declined substantially during the next 2 months.[38] Loss of β-carotene paralleled ascorbic acid and α-tocopherol losses, which indicates that depletion of natural antioxidants accelerates β-carotene losses. It has been proposed that tocopherols provide a first oxidation barrier, while ascorbic acid is required for tocopherol regeneration, and the carotenoids function as a second barrier against lipid oxidation.[84] Since levels of natural antioxidants play an important role in prevention of carotenoid degradation and color stability, several researchers have investigated the efficacy of supplemental antioxidant treatments. Addition of pepper seeds, which are rich in γ-tocopherol, to paprika and New Mexican chili powders has been shown to prevent color and carotenoid degradation.[39,60,62,63] Other treatments that have been used successfully to prevent color degradation during dehydration and storage of dried peppers include the synthetic antioxidant ethoxyquin, δ-tocopherol, and ascorbic acid.[85,86] In addition to antioxidant treatments, removal of oxygen in the package atmosphere by nitrogen flushing has been shown to stabilize color of dried red peppers.[87] Water activity and particle size of dried red pepper powders are also important considerations for carotenoid stability. It is recommended that coarse particles be used and powders dried to a water activity of 0.30.[87]

Chile powders are commonly treated with gamma irradiation as a microbial disinfection step. Color stability of dried red pepper powders was not affected by irradiation doses of 0, 1, 5, and 10 kGy, which indicates that carotenoids are relatively stable during irradiation.[88]

VII. CAPSAICINOIDS

Capsaicinoids are alkaloid compounds responsible for the hot flavor or pungency associated with consumption of *Capsicum* fruit. The hot flavor is due to structurally similar alkaloids or capsaicinoids (Figure 13.3). However, capsaicin and dihydrocapsaicin typically account for ~90% of the pungency of commonly consumed peppers.[7] Capsaicinoids are unique to the genus *Capsicum*, and their content varies greatly among species and cultivars due to differences in genetics, environmental growing conditions, and maturity.

Capsaicinoids are biosynthesized from L-phenylalanine and L-valine or L-leucine through vanillylamine and C_9 to C_{11} branch-chained fatty acids. The proposed pathway for synthesis of the vanillylamine moeity of capsaicinoids from L-phenylalanine is as follows: L-phenylalanine, *trans*-cinnamic acid, *trans-p*-coumaric acid, *trans*-caffeic acid, *trans*-ferulic acid, vanillin, and vanillylamine.[53] Unfortunately, many of the enzymes involved in capsaicinoid biosynthesis have yet to be identified. Capsaicinoids are reported to possess numerous pharmacological and medicinal properties, which have been reviewed extensively.[7,89] Capsaicin is commonly used as a treatment for pain and inflammation. Its mode of action is related to its interaction with transgeminal nerve endings, which release a neurotransmitter called substance P. Repeated capsaicin ingestion or topical application results in increased release of substance P, which results in its depletion, rendering the nerve endings unresponsive to subsequent applications. Due to its ability to alleviate a variety of human pain disorders, capsaicin has been used to prevent or alleviate many medical conditions including postmastectomy syndrome, urticaria, psoriasis, diabetic neuropathy, arthritis, osteoarthritis, pruritis, apocrine, chromhidrosis, contact allergy, postsurgical neuromas, reflex sympathetic dystrophy syndrome, vascular vestibulitis, shingles (*Herpes zoster*),

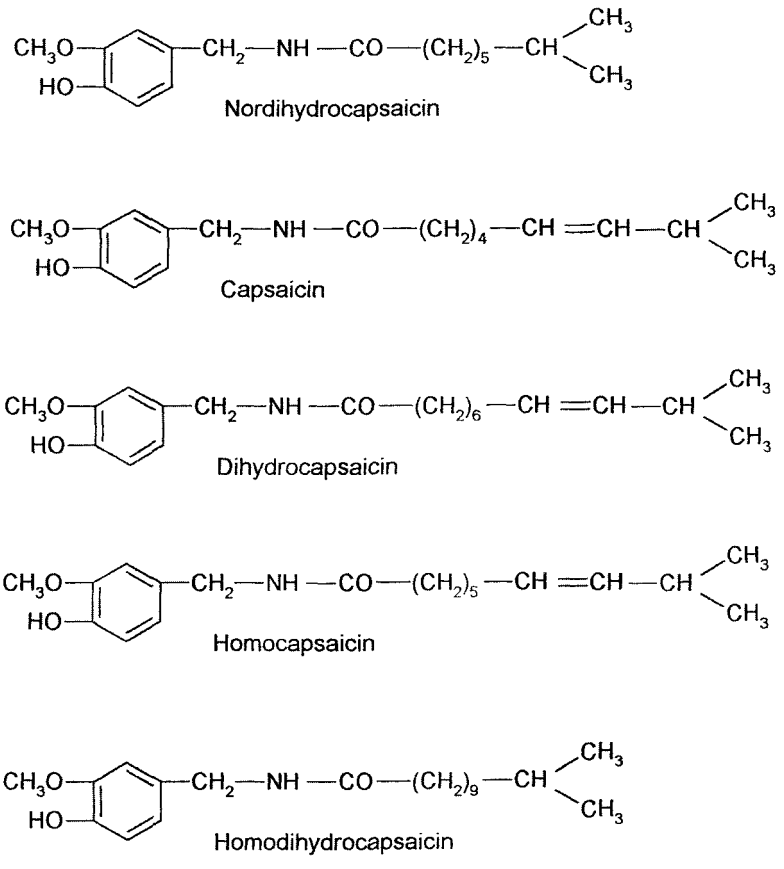


FIGURE 13.3 Capsaicinoids in *Capsicum* fruit.

cluster headaches, and urological disorders.[89,90] Capsaicinoids may also exhibit anticarcinogenic and antimutagenic properties. Several studies have shown that capsaicinoids may inhibit the metabolism and mutagenicity of chemical carcinogens.[91] In addition, capsaicinoids also have antioxidant,[17,92–94] antiplatelet, and anti-inflammatory activities,[95,96] and have been shown to induce apoptosis, an important step in carcinogen removal.[97]

The capsaicinoid content of *Capsicum* fruit is largely influenced by genetics, as shown in Table 13.5.[98–100] It is difficult to classify pepper species and types according to heat level because of the wide variation in heat level observed between cultivars. Total capsaicinoid content ranges from 720 to 4360 ppm for cayenne cultivars, 1110 to 7260 ppm for jalapeno cultivars, and 81 to 2690 ppm for New Mexican cultivars. Chile peppers may be classified according to level of pungency with 0 to 47 ppm being nonpungent, 47 to 200 ppm being mildly pungent, 200 to 1667 ppm being moderately pungent, 1667 to 4667 being highly pungent, and >5333 ppm being very highly pungent. According to the literature, bell-type peppers are nonpungent, while all other pepper types listed in the table vary considerably in degree of pungency. The unknown cultivars of "cascabel," New Mexican, and "pasilla" are the only peppers that fall within the mildly pungent category. Moderately pungent peppers include most New Mexican cultivars, several jalapeno cultivars, cayenne cv. Cayenne Short, cherry cv. Hot Cherry Pepper, *C. baccatum* cv. unknown, *C. frutescens* cv. Tabasco, and *C. pubescens* cv. unknown. Highly pungent peppers include most cayenne cultivars, "DeArbol" cv. Chile DeArbol, several jalapeno cultivars, New Mexican cv. NewMex Centennial Chile, several serrano cultivars, and "yellow mushroom" and *C. Cardenasii* cv. unknown. *C. chinense* cultivars and one jalapeno cv. JM are considered to be very highly pungent.

TABLE 13.5
Capsaicinoid Content of Fresh *Capsicum* Fruit

			ppm (dry weight)					
Species	**Type**	**Cultivar**	**Nordihydrocapsaicin**	**Dihydrocapsaicin**	**Homodihydrocapsaicin**	**Capsaicin**	**Total**	**Ref.**
annuum	Cascabel		7	42	ND	88	137	98
	Cayenne	Cayenne Short	140	270	—	310	720	99
		Cayenne Long Thick	430	1,100	—	1,200	2,730	99
		Carolina Cayenne	380	1,100	—	1,900	3,380	99
		Ultra Cayenne (Slim)	760	2,100	—	1,500	4,360	99
	Cherry	Hot Cherry Pepper	120	260	—	200	580	99
		Cubanella	55	291	18	834	1,198	98
	DeArbol	Chile DeArbol	280	620	—	1,100	2,000	99
	Jalapeno		107	595	28	1,307	2,037	98
		TAM	—	—	—	1,110	1,110	100
		TMJ	—	—	—	1,172	1,172	100
		T85	—	—	—	3,820	3,820	100
		JM	—	—	—	7,260	7,260	100
		M	360	1,200	—	1,900	3,460	100
		Early Jalapeno	190	840	—	770	1,800	100
	New Mexican	ND	42	ND	39	81	99	99

		Anaheim M	21	110	—	89	220	99
		Big Jim	14	100	—	160	274	99
		Sandia Hot	21	160	—	140	321	99
		Espanola Improved	28	190	—	250	468	99
		NewMex X Hot	54	480	—	640	1,174	99
		NewMex Cen. Chile	190	1,100	—	1,400	2,690	99
		Pasilla	18	144	7	195	364	98
	Serrano	Small Serrano	440	1,100	—	1,100	2,640	99
		Serrano	360	1,100	—	1,400	2,860	99
	Yellow mushroom		92	627	34	1,196	1,949	98
baccatum			79	352	12	558	1,001	98
cardenasii			706	934	67	984	2,691	98
chinense	Habanero		279	3,002	60	10,951	14,292	98
		Dom	250	5,200	—	14,000	19,450	99
			810	6,300	—	12,000	19,110	99
scotch bonnet	Chocolate		20	1,600	—	7,000	8,620	99
frutescens	Tabasco	Tabasco	47	370	—	670	1,087	98
pubescens			300	487	14	401	1,202	98
			412	441	22	502	1,377	98

In addition to genetics, pepper pungency is influenced by many factors, including fertilization practices, environmental growing conditions, application of bioregulators, and maturity. As demonstrated in Table 13.5, plant breeders have successfully developed pepper cultivars with varying levels of pungency. Genetic manipulation of pungency levels in peppers is important for various food and pharmaceutical applications. Development of heatless and mild jalapenos[101] has enabled processors of salsa and picante sauce to strictly control the pungency levels of their products. Mild, medium, and hot pungency levels in salsas are controlled through the addition of known amounts of capsaicinoid oleoresins. Conversely, plant breeders have developed pepper types with extremely high levels of capsaicinoids for use in skin ointments for pain relief, and for manufacturing aerosol sprays designed to incapacitate aggressive criminals.

Environmental stresses incurred during growth including excessive variation in water, temperature, and fertility generally lead to increased capsaicinoid synthesis. Peppers grown in hot weather generally have a higher capsaicinoid content than peppers grown in cool weather. Water deficit or excess will also induce stress and capsaicinoid synthesis. Extreme variation in capsaicinoid content may occur between plants grown in the same field due to differences in environmental conditions. New Mexican chile-type peppers grown in the same field in different plots differed by 400 ppm (6000 Scoville units) in total capsaicinoid content.[102] Fertilization practices have been shown to influence capsaicinoid content in pepper fruit. Plants receiving up to 48 kg/ha of phosphorus at varying levels of nitrogen (0, 80, 100 kg/ha) had elevated levels of capsaicinoids.[23] A combination of 100 kg/ha N_2 and 48 kg/ha P resulted in the highest capsaicinoid content. In another study, increasing levels of N_2 at the time of transplanting resulted in increased capsaicinoid content, with the highest level obtained at a rate of 15 m*M*.[103] Mineral supplementation of padron peppers with 0.1 g of 13N–40P–13K during vegetative growth and 15N–11P–15K during flowering also resulted in increased capsaicinoid content.[104] Plant age also influences capsaicinoid content. Capsaicinoids typically reach maximum levels around 28 to 50 days after flowering, and then levels stabilize or decline gradually as fruit ripens.[105,106] Thus, immature fruit within the same plant may have higher capsaicinoid content and heat levels than more mature fruit. Loss of capsaicinoids during pepper ripening may reflect increased peroxidase activity.[106] A peroxidase isoenzyme B6 was shown to oxidize phenolic precursors of capsaicin, indicating that phenylpropanoid intermediates of capsaicin biosynthesis may compete with capsaicin for synthesis of lignin-like substances in the cell wall.[107]

Application of bioregulators may also affect capsaicinoid content. Ethephon applied at 1000 and 3000 µl/l increased capsaicinoid content of cayenne peppers by 50%.[108] However, the treatment does not appear to be commercially feasible due to fruit damage and yield loss.

Effects of Postharvest Handling and Processing

Capsaicinoid content of pepper fruit is affected by food-processing conditions. Freezing and canning of jalapeno peppers resulted in total capsaicinoid losses of 43.8 and 33.1%, respectively, while cooking resulted in an increase of 19.2%.[109] Loss of capsaicinoids may be attributed to leaching during water blanching, residual enzyme activity, or liberation from complexed compounds during pasteurization. In another study, losses of capsaicin and dihydrocapsaicin in pasteurized yellow wax peppers after 4 months storage were 30 and 10%.[34] Calcium chloride brine treatment, which is commonly used as a firming agent, did not affect capsaicinoid losses in pasteurized yellow wax peppers stored for 4 months. In contrast, the capsaicinoid content of jalapeno peppers was unaffected by blanching and pasteurization steps.[35]

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14 Modification of Atherogenesis and Heart Disease by Grape Wine and Tea Polyphenols

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I. INTRODUCTION

Wines and teas have a long history of being touted for the promotion of human health and for the treatment of disease. With recent legislation now allowing wineries to add a general statement of the health benefits of grape wine to their label, what is it about these beverages that has given them recognition over the years as a health-promoting food or nutraceutical?

Over the past two decades a number of epidemiological studies investigating incidences of myocardial infarctions, angina pectoris, or coronary-related deaths have reported that they were inversely related to moderate alcohol intake.[1–15] These studies examined subjects ranging in age from 25 to 84 years old and involved hundreds to thousands of people in a number of different countries. Further analyses revealed that this negative association was not truly linear, but followed

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a U- or J-shaped curve.[11,15–17] That is, at low to moderate ethanol intake, the risk of heart disease or death is lower than in abstainers, but at high intake levels, these risks rise again. Although the mechanisms for this reduced risk are not well understood, ethanol intake has been reported to raise the plasma levels of high-density lipoproteins (HDL) and/or lower the levels and rate of oxidation of low-density lipoproteins (LDL).[3,18–20] Ethanol intake is also known to prolong the clotting times of blood.[21,22]

This association between moderate alcohol consumption and risk of ischemic heart disease has caught the public's attention in what has been labeled the "French Paradox." Epidemiological studies have observed that in southern France mortality rates from heart disease were lower than expected despite the consumption of diets high in saturated fats and the tendency to smoke cigarettes.[22,23] These coronary-related deaths in France were reportedly about one third the rate in Great Britain, and lower than any country examined except for China and Japan, where diets are generally low in saturated fats.[22] Both dietary[8,24,25] and nondietary factors such as lower levels of stress,[26] underreporting of deaths,[27] and, most recently, a time lag association, similar to that observed between cigarette smoking and incidence of lung cancer in women,[28] have been proposed to explain this so-called paradox.

Nevertheless, in addition to their Mediterranean style diet, the most attention in explaining the French paradox has focused on the common practice of the French consuming wine, particularly red wine, with their meals.[4,7,8,25] France has the highest per capita consumption of grape wine of any other developed country.[25,26] Indeed, epidemiological studies suggest that the consumption of wine at the level of intake in France could explain a 40% reduction in heart disease.[22] However, it should be noted that this relationship does not appear to hold for other regions of France and overall longevity and mortality rates from all causes in France are similar to those in other industrialized countries.[25]

Epidemiological studies evaluating the protective effect of drinking tea from the development or incidence of cardiovascular disease are far fewer in comparison to the number of studies examining ethanol intake. Nevertheless, tea consumption is reported to have similar protective effects.[29–32] For example, a study in men and women 30 to 49 years old found that tea consumption was inversely related to serum cholesterol levels and systolic blood pressure and there was a slightly, but not significantly, lower mortality in those individuals who drank one or more cups of tea per day compared to those who drank less than a cup per day.[32] In addition, a recent study[33] in Japan noted that green tea consumption was directly related to lower serum cholesterol concentrations, higher HDLs, and lower LDLs. In contrast, a British study[34] saw no inverse relation between tea consumption and coronary heart disease. Also, in healthy adults drinking black tea for 4 weeks, statistically significant effects on serum cholesterol, HDL, LDL, or triglycerides were observed only in individuals with specific atherogenic apoE genotypes.[35]

Although the exact mechanisms by which wine or tea consumption could offer protection against atherosclerosis and ischemic heart disease are not fully known, a large body of literature has emerged which suggests that the actions of polyphenolic compounds found in these beverages may account for this protection.[36–39] Table 14.1 lists the various actions suggested through which these compounds could impact on the development of cardiovascular diseases (CVD). This chapter discusses these polyphenolic substances, the epidemiological evidence to support these claims, and the evidence for the proposed mechanisms through which these substances may reduce certain risk factors associated with development of CVD.

II. POLYPHENOLS

A. Chemical Background and Nomenclature

Wine, grapes, and tea are known to contain a variety of polyphenolic compounds.[40–47] The terms *polyphenols* and *phenolic* are all-encompassing, ranging from simple phenolic acid to polymerized

TABLE 14.1
Proposed Properties of Wine and Tea Polyphenols to Reduce Risk of Atherosclerosis or Heart Disease

I. Effects on Plasma Lipids
- Increase HDL levels
- Decrease LDL levels
- Inhibit lipoprotein synthesis
- Decrease lipoprotein [a] levels

II. General Antioxidant Activity
- Chelate transition metals
- Inhibit oxidation of LDL
- Scavenge oxygen free radicals

III. Other Effects
- Anticoagulant effects including aspirin-like activity
- Enhance NO synthesis to keep blood vessels patent
- Other anti-inflammatory activity

compounds like tannins. Overall in the plant kingdom, polyphenols or phenolic compounds account for well over 4000 individual compounds.[37,40,43–45] These compounds are the secondary by-products of plant metabolisms and their large numbers are indicative of what can arise from various hydroxylation, methoxylation, glycosylation, and acylation reactions during their biosynthesis. Consequently, in addition to wine and tea, polyphenols are found in many commonly eaten fruits and vegetables, such as grapes, apples, grapefruit, onion, eggplant, and kale, as well as herbs, spices, cereals, legumes, and nuts.[37,45]

Polyphenols have generally been classified in three major groups: (1) simple phenols and phenolic acids, (2) flavonoids, and (3) hydroxycinnamic acid derivatives,[37] although other groupings have been described.[45] Many of the compounds found in tea and wine are low-molecular-weight polyphenols such as flavonoids,[40–47] also loosely referred to as bioflavonoids.[40] Many flavonoid compounds occur as sugars (glycosides) and tend to be water soluble. They play significant roles in the plant kingdom. Many flavonoids, especially the flavanols, are astringents, whereas others have evolved to protect plants against microbes, parasites, and oxidative injury.

The flavonoids are based on the flavan nucleus consisting of 15 carbons within three rings recognized as A, B, and C (Figure 14.1A).[40,43 45] The basic structure is a phenyl benzo(γ)pyrone derivative. The differences between the various subclasses of polyphenolic compounds are due to the presence of 3-hydroxyl and/or 2-oxy groups, the number of hydroxyls in the A and B rings, and the absence/presence of double bonds in the pyrane ring. The chemical substitutions and structures that define the various flavonoids have been reviewed by Bravo.[45] Flavonoids may occur as monomeric, dimeric biflavonoids (not to be confused with bioflavonoids) or oligomeric compounds. Tannins (Figure 14.1B) are polymeric derivatives that are classified into two groups: (1) condensed (proanthocyanidins) or (2) hydrolyzable which often contain gallic acid. An example is epicatechin gallate (ECG), shown in Figure 14.1C, often found in teas.

As might be anticipated because of the large spectrum of compounds that can be listed as polyphenols or flavonoids, there is a lack of agreement on nomenclature and classification. Using chemical structures, flavonoids can be subdivided into flavonols, flavones, flavanones, flavanols (catechins), anthocyanidins, isoflavones, dihydroflavonols, and chalcones.[45] Another classification system uses the phrase "minor flavonoids" to include flavanones, flavanols, and dihydroflavonols or those flavonoids with limited natural distribution.[48] With respect to mammalian biological activity, much of current interest in flavonoids is related to the 4-oxo-flavonoid structures, i.e., flavonols, flavones, flavanones, isoflavones, and dihydroflavonols.[45] Flavonols, flavones, and anthocyanidines are second only to the carotenoids with respect to being compounds of vivid color.[43–45]

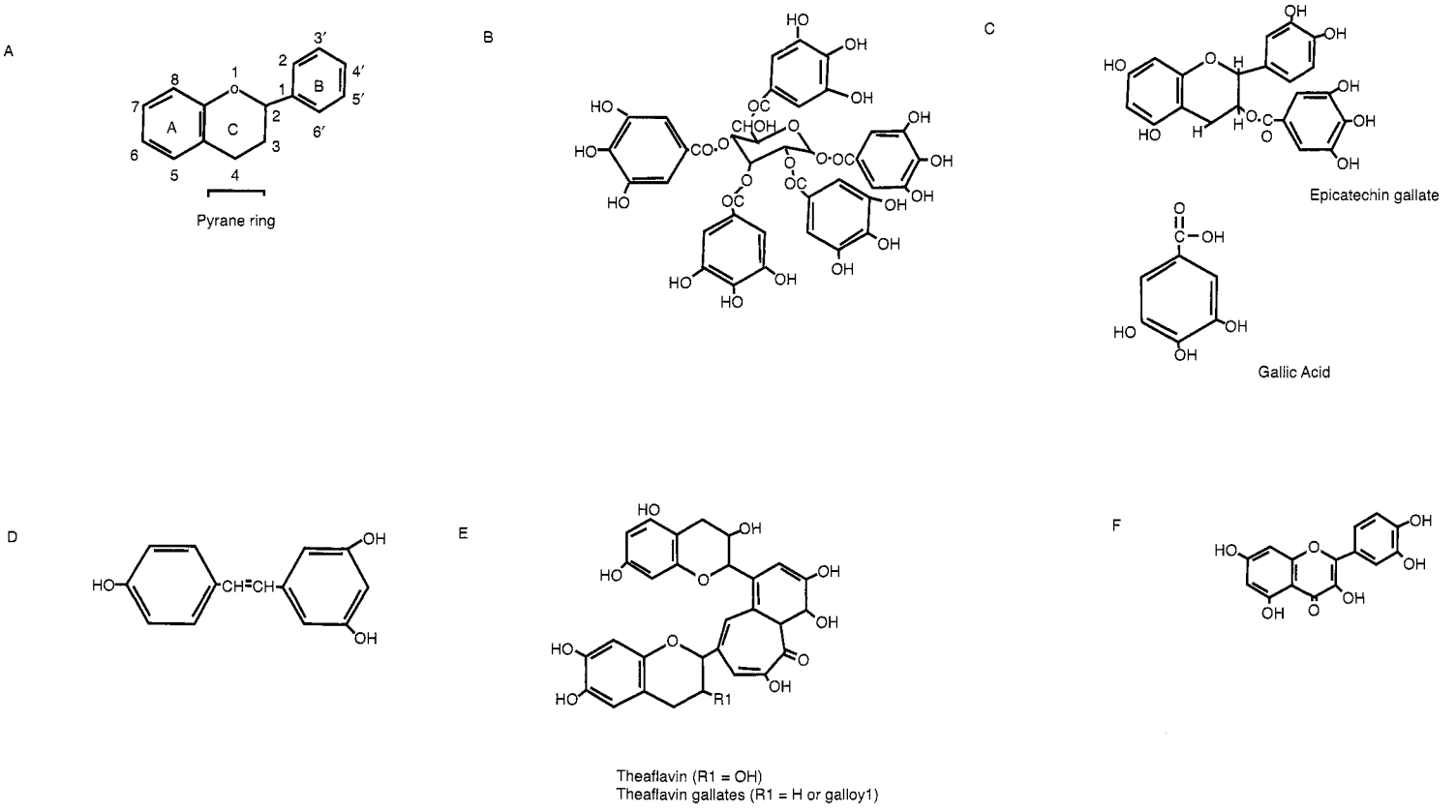

FIGURE 14.1 Select flavonoids from wine and tea. (A) Flavonoid base structures with carbon numbering; (B) Tannin chemical structure; (C) Epicatechin gallate and gallic acid chemical structures; (D) Resveratrol (3,5,4′-trihydroxystilbene) structure (resveratrol can exist in the *cis* or *trans* configuration); (E) Theaflavin and theaflavin gallate (example of flavonoid oxidation by-product); and (F) Structure of quercetin.

Although our infatuation with flavonoids as potential health promoters seems recent, over a dozen flavonoid-containing medicinals have been known and used in traditional medicine.[49] A number of plant species, because of their natural content of flavonoids, have been used throughout the world for various medicinal purposes. They have been used as anti-inflammatory, antiseptic, antiarrhythmic, antispasmodic, and anxiolytic agents, as sedatives, and for wound healing, to name a few. As a group, the polyphenols have generally been recognized to possess significant antioxidant activities ranging from scavenging oxygen free radicals to chelating metals.[50–52]

B. Polyphenols in Wines and Grapes

The polyphenols in wine include phenolic acids, a number of flavonoids (anthocyanins, tannins, caffeic acid, rutin, catechin, myricetin, quercetin, epicatechin), and nonflavonoids. Proanthocyanidins, polymers or oligomers of catechin units, are the major polyphenols in red wine and especially in grape seeds.[53,54] Grape skins and juice contain anthrocyanins and flavonoids (e.g., quercetin and myricetin).[55] Nonflavonoids are derivatives of cinnamates, tyrosol, volatile phenols, and hydrolyzable tannins.[53] Of the nonflavonoids in wine, resveratrol has sparked much interest for its potential health-enhancing effects.[52] Besides grapes, only a few other plant species, such as peanuts, contain resveratrol.[55] These stilbenes and stilbene glycosides have antifungal activity[52] and their health benefits have been attributed to their phytoestrogen properties,[52,55,56] to metal ion chelation,[57] or to general antioxidant activity.[50,52,55,56]

The total polyphenolic content of red wines has been estimated to be about 1200 mg/l,[58] while others have reported concentrations as high as 4000 mg/l, compared with white wine with about 200 to 300 mg/l.[46] It is also reported that flavonoid content of red wine can be about 20-fold higher than in white wine,[58] while grape juice has about one half the flavonoid content of red wine by volume.[16] For example, the concentrations of epicatechin and related compounds in wine have been estimated at 150 and 15 mg/l for red and white wine, respectively.[59] It has also been estimated that quercetin concentrations in wine are about 25 mg/l. Nonflavonoids, such as hydroxybenzoate and hydroxycinnamate, do not differ signficantly between red and white wine.[58] Resveratrol (3,4′,5-trihydoxystilbene; Figure 14.1D) is present mainly in grape skins and, therefore, is found primarily in red wines, with concentrations around 1 mg/l.[59] The concentrations of select flavonoids and resveratrol in wine are summarized in Table 14.2.

It is also realized that aged wines differ in the nature of their polyphenols compared with young wines or, for the most part, those found in grape juices.[45,53,60] Phenolic concentrations in wine

TABLE 14.2
Concentrations of Select Flavonoids and Resveratrol in Wine

Subclasses of Flavonoid	Compound	Quantity, mg/l	
		White Wines	Red Wines
Flavonols			
	Myricetin	0	8.5
	Rutin	0	9
	Quercetin	0	7–10
Flavanols		56	274–450
	Catechin	35	191–360
	Epicatechin	21	82–100
Anthocyanins			
	Anthocyanidin	0	2.8
Resveratrol		0.027	1.5

Source: From References 46, 53, 59, and 83.

increase during skin fermentation and decrease as phenols interact with proteins and yeast cell membranes and precipitate. Wine aging results in further modification in the phenolic content. In addition, herbicides and insecticides are known to modulate the concentration of polyphenolic compounds and secondary compounds through reduction of carbon fixation in plants. In summary, the amount of flavonoids in wine can be influenced by several factors, including temperature, sulfite and ethanol concentrations, the type of fermentation vessel, pH, and yeast strain.[53,56] However, if open wine is protected from light, the polyphenols appear to be stable for about 1 week at room or refrigeration temperatures.[61]

C. Compounds Found in Teas

Tea is second only to water as the most-consumed beverage in the world.[40] Worldwide per capita consumption of tea is estimated at about 0.12 l/day, but in some locations it can be up to 5 l/day.[42,62] Tea is the beverage originating from leaf of the plant *Camellia sinensis* and its numerous varieties. The tea leaves contain more than 35% of their dry weight in polyphenols. Breeding and selection have resulted in the hybridization and emergence of thousands of types of teas with varying properties and composition.

Green tea is the product produced from fresh leaf. Rapid inactivation of the enzyme, polyphenol oxidase, by steaming or rapid pan-firing, rolling, and high temperature air-drying, is used to make green tea in Japan and China, and preserves the polyphenol content. Thus, green tea is rich in the flavanols catechin, epicatechin, ECG, gallocatechin, epigallocatechin (EGC), and epigallocatechin gallate (EGCG), the flavanols that have generated the most human interest.[42,62] It has been estimated that one cup of green tea can contain 100 to 200 mg catechins.[63] In general, green tea contains higher concentrations of the catechins than wine. In addition, green tea contains quercetin, kaempferol, myricetin and their glycosides, apigenin glycosides, and lignans, but at lower concentrations.[64,65] A summary of the most common flavonoids in teas are presented in Table 14.3.

Black tea is derived from aged tea leaves that have undergone enzymatically catalyzed aerobic oxidation and chemical condensations, particularly of the catechins. Consequently, catechin levels

TABLE 14.3
Concentrations of Phenolic Acid and Flavonoids and Their Oxidation Products in Tea

		Quantity, mg/g	
Subclasses of Flavonoid	**Compound**	**Green Tea**	**Black Tea**
Flavonols		50–100	60–80
	Quercetin		10–20
	Kaempferol	20–45	14–16
	Myricetin		2–5
Flavanols		300–400	50–100
	Catechin	10–20	5
	Epicatechin	10–50	10–20
	Epigallocatechin (EGC)	30–100	10–20
	Gallocatechin	10–30	
	Epicatechin gallate (ECG)	30–100	30–40
	Epigallocatechin gallate (EGCG)	70–150	40–50
Flavandiols		20–30	
Phenolic acids		30–50	100–120
Theaflavins			30–60
Thearubigens			300–600

Source: From References 40, 41, 54, 86, and 185.

are lower in black than in green tea. Interestingly, in food science, oxidation properties of catechins have been adopted for use as food antioxidants similar to that of butylated hydroxyanisole (BHA).[66] The principal products of catechin oxidation are the quinones, which in turn form seven-membered ring theaflavin or theaflavin gallate compounds, shown in Figure 14.1E, as well as thearubigins.[67] Theaflavin concentrations are directly correlated with black tea quality. Theaflavins (1 to 2% by dry weight) are mostly responsible for the reddish color and astringency of black tea. Partial oxidation of green tea results in oolong tea, which retains much of the original polyphenol content of the leaf.

D. Absorption and Metabolism of Polyphenols

Crucial to any discussion regarding the efficacy of wine and tea polyphenols in the prevention of atherosclerosis and heart disease is how well such compounds are absorbed through the intestinal tract wall, how well they are distributed into various tissues, especially blood plasma, and their metabolism and rate of elimination. Unfortunately, there is limited information in humans, which has led to uncertainty that these compounds could express *in vivo* antioxidant activity of physiological significance. Because such compounds occur as complex mixtures in plant materials and their enormous variability, it is difficult to study bioavailability and physiological effects. Polyphenols only sparingly occur in the free form.

Earlier studies in the United States estimated that the daily intake of flavonoids was about 1 g/day when expressed as glycosides, or 650 mg/day when expressed as aglycones.[68] Hollman and colleagues[39] however, have questioned these values as too high, and others have estimated that the average intake of all flavonoids from dietary sources is between 23 and 170 mg/day.[29,69] In a Dutch study, daily intake of all flavonoids was estimated at 23 mg/day with quercetin accounting for 16 mg/day.[29,69] This is in keeping with the observation that, of the flavonoids, quercetin is generally found in the highest concentration in food. Its concentration in grapes is reportedly 1.4 mg/kg, whereas green tea contains >10,000 mg/kg quercetin glycosides and kaempferol.[70] In addition, Hollman and colleagues[39] summarized the average daily flavonol intake from six studies as 4 to 68 mg/day. Interestingly, on a mg/day basis, flavonoid intake exceeds the average daily requirement of vitamin E and β-carotene.

The absorption of polyphenols varies depending on the type of food, the chemical form of the polyphenols, and their interactions with other substances in food, such as protein, ethanol, and fiber. As an example, quercetin absorption was 52 ± 15% from quercetin glucosides in onions, 17 ± 15% from quercetin rutinoside, and 24 ± 9% from quercetin aglycone.[70] Urinary excretion was about 0.5% of the amount absorbed. Flavonoids, such as quercetin, can be absorbed either as the free aglycone or glycoside as demonstrated by detection in blood and urine following consumption of nonsupplemented diets.[44,71,72] It has also been reported that polyphenols from wine may be absorbed better than the same substances from fruits and vegetables, because the ethanol may enhance the breakdown of the polyphenols into smaller products that are absorbed more readily.[38]

The bulk of such compounds is found as plant glycosides. Therefore, they were expected to remained unchanged in most of the gut, since it was presumed that humans lack the appropriate β-glycosidases to metabolize the sugars. Data, however, suggest that glycosidases from bacteria that colonize the ileum and cecum are involved in the breakdown of flavonoids. For example, it has been shown that flavonoid glycosides ingested by germ-free rats are recovered intact in the feces.[73] Others have found that the administration of 0.5 g/day of catechin or tannic acid to rats over a 3-week period resulted in less than 5% excreted unchanged in the feces.[74] Glycones from onions have been shown to cross the mucosal layer of the intestinal cells, suggesting that humans may have hydrolases to remove sugar components to form aglycones.[75] However, it remains uncertain if the hydrolysis of flavonoid glycosides is necessary for absorption in humans. Also, further research is needed to confirm that deglycosylation of flavonoids can occur independent of gut microbial action.

Nevertheless, studies in experimental animals and humans indicate that some polyphenols, at least, can be absorbed. In rats fed up to 16.4 mmol/kg diet quercetin for 10 days, plasma concentrations were over 100 μM.[76,77] In animals and human studies, between 10 and 20% of an oral dose of quercetin is absorbed.[78] After tea drinking, only 0.5% of the quercetin was excreted unchanged.[79] These authors concluded that plasma concentrations of quercetin and kaempferol reflected short-term intake. In general, peak blood levels of flavonoids occur between 2 and 3 h after consumption and the elimination half-life varied between 5 and 17 h depending on the particular flavonoid or the food source.[80,81] In addition, a recent study reported that in rats fed red wine containing 6.5 mg/l of resveratrol for up to 15 days, some of the intact compound was detected in plasma and tissues, but the concentrations found were considered lower than would be expected to be physiologically important.[82] However, it remains to be determined whether repeated intake would increase these tissue levels further.

Clifford and colleagues[83] detected catechin in plasma from mice fed a diet containing red wine solids, and in human subjects, EGCG appeared in plasma 30 min after drinking 300 ml of green tea.[84] Studies with EGCG found that in adult men drinking decaffeinated green tea containing 88 mg of EGCG and 82 mg of EGC, plasma concentrations 1 h after ingestion ranged from 46 to 268 ng/ml for EGCG and from 82 to 206 ng/ml for EGC.[85] It was also found that addition of milk to black tea did not affect catechin absorption and after a single tea consumption, the half-life of catechins in blood varied from 4.8 h for green tea to 6.9 h for black tea.[86] However, some studies of polyphenol absorption and metabolism may be misleading due to administration of pharmacological doses (4 g) in some human studies.[87] Handling of pharmacological doses may not reflect the normal physiological mechanisms of absorption and metabolism of dietary flavonoids that would occur with usual dietary intakes.

Studies also indicate that the liver is the primary site of polyphenol metabolism, although other sites such as kidney or intestinal mucosa may be involved. In the liver, these compounds can undergo (1) methylation, (2) hydroxylation, (3) reduction of the carbonyl group in the pyrane ring, and (4) conjugation reactions. The most common degradation pathway for flavonoids is through conjugation with glucuronides or sulfate.[88] In addition, some flavonoid metabolites can be recycled via the enteroheptic biliary route.

III. EPIDEMIOLOGY OF POLYPHENOLS AND ATHEROSCLEROSIS

Evidence that dietary flavonoid intake was inversely related to mortality from coronary heart disease has been supported by recent epidemiological studies.[29,31,89–91] In the Zutphen Elderly study, Hertog and colleagues[29,90] showed that after adjustment for age, weight, certain risk factors of coronary artery disease, and intake of antioxidant vitamins, the highest tertile of flavonoid intake, primarily from tea, onions, and apples, had a relative risk of heart disease of 0.32 compared with the lowest tertile. Although the magnitude of relative risk was less in a Finnish study, the data were similar to that observed in the Dutch study.[31] It should be noted that tea and grape wine consumption is rather low in Finland.

However, not all studies have seen protective effects. A U.S. study of a large cohort of male health professionals did not observe such a negative correlation between flavonoid intake and incidence of coronary heart disease, although there was a tendency for protection in men with established heart disease.[91] In addition, a Welsh study[92] observed higher mortality from heart disease associated with high flavonol intake, primarily from tea consumed with milk. In this study, however, it was noted that tea consumption was associated with a lower social class and a less healthy lifestyle, which included cigarette smoking and a higher fat consumption. In contrast, tea consumption in the above Dutch studies was associated with a higher social class and healthier lifestyle. Thus the evidence supporting a protective effect of polyphenol intake against ischemic heart disease is suggestive, but still inconclusive. The question then arises, what is our understanding of the atherogenic process that could identify areas where polyphenols could exert a beneficial effect?

IV. ETIOLOGY OF ATHEROSCLEROSIS

Although the etiology of atherosclerosis and the development of heart disease are complex, it is generally agreed that the process of atherosclerosis begins with the accretion of soft fatty streaks along the inner arterial walls. As we age, the fatty streaks enlarge and become stiffer, and mineral deposition occurs with development of the well-recognized arterial plaque. Platelets respond to plaque as they normally do to vessel injuries. Thus, blood clots may stick to plaque, grow, and restrict blood flow, producing thrombosis. In addition, clots may break free from the artery wall and eventually lodge in a small artery producing an embolism, shutting off the blood supply to tissues.

It is now hypothesized that blood cholesterol is linked to atherosclerosis and the risk of ischemic heart disease by its presence in LDL cholesterol.[93] Although the mechanisms through which high plasma LDL concentrations increase the risk of CVD are not completely understood, evidence is emerging to implicate the oxidation of LDL by free radical by-products or via the process of oxidative injury as an important factor.

Atheromatous lesions occur in the subendothelial space of the arterial vessel because of the deposition of cholesteryl esters in macrophages and smooth muscle cells, forming foam cells (Figure 14.2).[94] Usually, macrophages have few LDL receptors, which, coincidentally, are downregulated as native plasma LDL concentration increases. However, it was found that chemically modified or acetylated LDL can be recognized by specific scavenger receptors on the macrophages. As such, the modified LDL is endocytosed at a higher rate than native LDL and is not regulated. This results in overaccumulation of LDL in subendothelial cells and the initiation of atherosclerosis. In addition, oxidized LDL is also chemotactic for macrophages and monocytes. Thus, altered LDL initiates

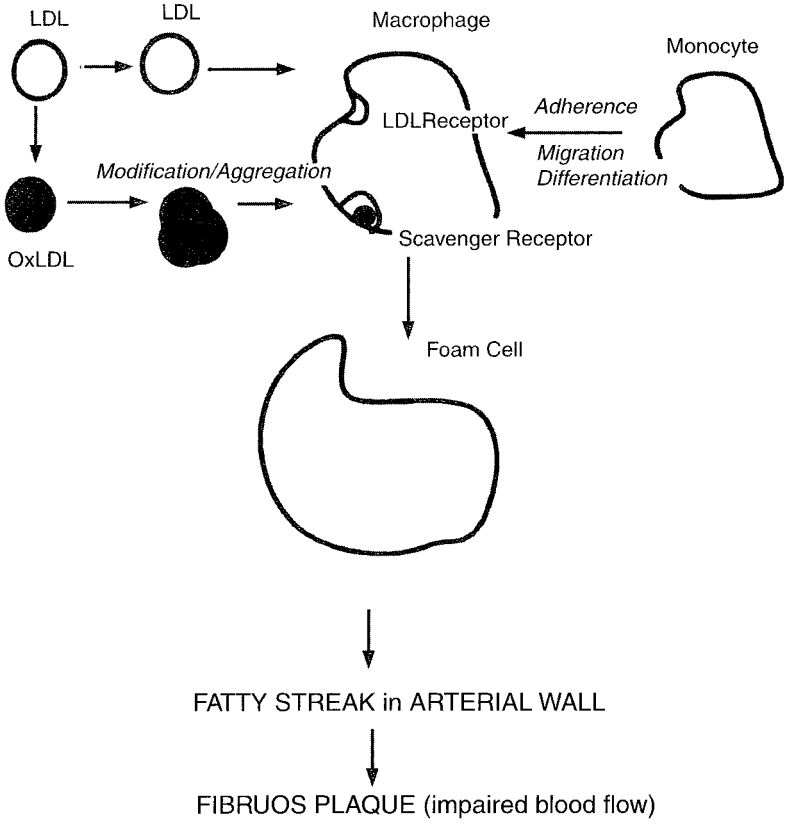

FIGURE 14.2 Process of LDL oxidation and initiation of atherosclerosis.

and promotes atherogenesis. They are responsible for the accumulation of cholesterol in atheromatous lesions, prevent reverse cholesterol uptake by HDL, and due to their chemotactic properties, promote adhesion of platelets to the endothelium, thereby advancing atherosclerotic lesion progression. Alternatively, LDL aggregates, another modification of LDL, are taken up by macrophages at increased rates, also leading to foam cell formation. These potential mechanisms have generated the oxidant theory of atherogenesis and have prompted the investigation of antioxidants as therapy or preventive measures.[93,95–101]

Like other lipid components, such as cell membranes, LDL particles contain several compounds with antioxidant capabilities: vitamin E, β-carotene, and other carotenoids. Several reports have shown an inverse relationship between dietary intakes of antioxidants and LDL oxidation as well as CVD. The simultaneous presence of several antioxidants has been suggested as advantageous because since such compounds would naturally occur at relatively low concentration *in vivo*, the addition of polyphenols would augment or enhance the efficiency of endogenous antioxidants, like vitamin E.[102] Thus, flavonoid compounds could impact significantly in reducing the overall oxidative stress within the body. However, all antioxidants are not equivalent in their ability to prevent LDL oxidation. When human subjects are given a regimen of greater than 25 IU/day of vitamin E, the antioxidant inhibits the oxidation of LDL.[97] Evidence is accumulating to suggest that vitamin E may reduce clinical events associated with CVD,[98] whereas studies with β-carotene have not been as protective.[99–101]

V. ACTIONS OF POLYPHENOLS ON RISK FACTORS ASSOCIATED WITH CVD

A. Effects on Cholesterol

As noted above, several studies in humans have suggested that the consumption of wine or ethanol was associated with lower serum cholesterol and LDL levels, and higher HDLs.[9,10,103] Also, wine was observed to be more effective than ethanol in preventing the development of atherosclerotic lesions in cholesterol-fed rabbits.[104]

It was also observed that red wine consumption decreased plasma concentrations of lipoprotein [a],[105] identified as an independent risk factor for atherosclerosis.[106] In contrast, another clinical study failed to observe an effect of red wine consumption on lipoprotein [a] concentrations.[107]

Tea consumption has also been associated with decreased serum triacylglycerols and cholesterol in humans.[40,108] The potential significance of these observations with respect to CVD comes from animal studies. For example, in rabbits fed a high-fat diet, green, but not black, tea consumption reduced aortic lesion formation by 31% compared with controls.[109] Green tea given to hypercholesterolemic rats and spontaneously hypertensive animals lowered blood cholesterol and pressure, respectively.[40] In mice fed an atherogenic diet, green tea extract prevented the increase in serum and liver cholesterol levels observed in controls.[110] Some studies suggest that the protective effects of tea, such as decreasing LDL cholesterol and increasing HDL cholesterol, seem to be correlated best with green rather than black tea,[33,111] suggesting an important role for the catechins. As an example, Xu and colleagues[112] reported that in hamsters fed a hypercholesterolemic diet for 16 weeks catechin supplementation was as effective as vitamin E in inhibiting plaque formation. Other work in hamsters suggests that the hypolipidemic activity of green tea catechins relates to their action on the absorption of dietary fat and cholesterol, rather than inhibitory effects on cholesterol or fatty acid synthesis.[113]

B. General Antioxidant Effects

It is likely that various polyphenols, including flavonoids, act similarly to dietary antioxidants and that collectively they may bestow protection from the development of heart disease. Physical and chemical properties of individual polyphenolic compounds impact strongly on their abilities to be

potent antioxidants, and these properties have been well described.[50,114] Such compounds are capable of reacting with a variety of potential disease-promoting free radicals, including superoxide, hydroxyl, peroxyl, alkoxyl, and nonradical species, e.g., singlet oxygen, peroxynitrite, and hydrogen peroxide.[50,114,115] It has been proposed that quercetin (see Figure 14.1F) possesses many of the properties considered essential for the ideal antioxidant.[45,50]

A number of *in vitro* studies have supported the idea that wines possess intrinsic antioxidant activity. Maxwell and colleagues[116] observed that red wines had about 30-fold greater antioxidant activity than normal human serum. It was also observed that the total reactive antioxidant potential of red wines was six to ten times higher than white wine.[117] The contribution of various polyphenols to the total antioxidant activity of red wine has been reported.[114]

Both green and black tea also exhibit significant antioxidant potential. For example, black tea extracts could prevent *in vitro* lipid peroxidation of human red blood cell (RBC) membranes and whole RBCs better than pure catechins in these systems.[118] In addition, green tea polyphenols have also been observed to scavenge peroxynitrite by preventing tyrosine nitration.[119,120] It also appears that adding milk to tea resulted in significant loss of tea antioxidant activity,[92] likely because of complex formation of tea polyphenols with milk proteins. The antioxidant activity of tea relative to other fruits and vegetables has been summarized by Prior and Cao.[121] Yet, controversies remain. It was reported that green and black tea have a greater antioxidant index than grape juice or wine.[122] Serafini and colleagues[123] reported that the *in vitro* antioxidant activity of black tea was 3 to 4 m*M*, or about one third the activity reported for red wine,[116] but the contribution of alcohol to these values is not fully known.

Antioxidant properties of wine have also been observed *in vivo*. Whitehead and colleagues[124] fed nine healthy subjects 300 ml of red wine and observed 18 and 11% increases in serum antioxidant capacity after 1 and 2 h, respectively, compared with 22 and 29% increases at these times in subjects who took 1000 mg ascorbic acid. Lesser increases in serum antioxidant capacity were observed if the subjects drank white wine, or apple, grape, or orange juice. However, Durak and colleagues[125] observed that plasma antioxidant potential was about 20% higher than baseline 4 h after normal subjects ate 1 g/kg of black grapes. Others observed that drinking red wine with meals or consumption of red wine polyphenols (1 or 2 g/day) increased total plasma antioxidants by 11 to 15%, respectively, in comparison to a 7% increase by vitamin E.[116,126,127] Struck and colleagues[128] observed an antioxidant effect of wine, defined as a reduction in thiobarbituric acid reactive substances, in 20 subjects with hypercholesterolemia who drank 180 ml/day of red or white wine for 28 days. In contrast to other studies, they observed a greater effect when the subjects drank white wine compared with red wine. In addition, they observed no changes in plasma vitamin E, vitamin C, or β-carotene, but consumption of either wine resulted in a 23% reduction from baseline in plasma retinol levels. Although these results suggest that the enhanced antioxidant potential observed after drinking wine is not due to higher concentrations of plasma antioxidant vitamins, a study by Day and Stansbie[129] reported that 73% of the increase in serum antioxidant capacity following consumption of port wine in 6 individuals could be attributed to an increase in serum uric acid levels, a well-recognized antioxidant.[130] On the other hand, Cao and colleagues[131] observed an 8% increase in serum antioxidant capacity in elderly women who drank 300 ml of red wine, which could not be ascribed to an increase in uric acid or vitamin C. Others have demonstrated that both red and white wine could also inhibit hydrogen peroxide–induced DNA damage in human lymphocytes[132] or could decrease the amount of unstable acetaldehyde–albumin complexes in individuals drinking excessive amounts of wine.[133]

As mentioned, tea polyphenols have been shown to have significant antioxidant activity *in vitro*.[121] However, few studies have examined the *in vivo* effects of tea consumption on plasma antioxidant activity. Serafini and colleagues[123] observed a 41 to 48% increase in plasma antioxidant activity in subjects who drank tea. Peak activity occurred at 30 and 50 min after green or black tea consumption, respectively, and returned to baseline after 80 min. In addition, green tea consumption appeared to reduce DNA markers of oxidative stress in smokers more than in nonsmokers.[134]

It should be noted that studies that did not observe an effect of red wine on plasma antioxidant status may reflect too low a consumption[105] or, as in a rat study, may reflect the limited effects polyphenols may exert when a well-balanced diet with more than adequate intake of micronutrients is consumed.[135] Thus, from the above studies it would appear that polyphenols in wine and tea demonstrate antioxidant activity, but the expression of this activity can depend on a variety of dietary and other health-related factors.

C. LDL Oxidation

Most flavonoids found in teas and wines have a lower oxidation potential than the vitamin E radical. Therefore, analogous to ascorbic acid, flavonoids have the ability to reduce vitamin E radical or to recycle vitamin E as an antioxidant. This is significant in LDL oxidation, because vitamin E represents the first line of defense against LDL oxidation.[97] Once vitamin E is exhausted, the LDL is no longer protected, unless vitamin E can be recycled by appropriate reducing agents, e.g., flavonoids. Evidence that the flavonoid caffeic acid can increase plasma and lipoprotein vitamin E levels has been observed in rats.[136] Furthermore, flavonoids can chelate divalent metal ions and prevent such metal ions from initiating oxidation. Finally, flavonoids may protect vitamin E in lipid oxidation by being oxidized themselves in preference to vitamin E, or delaying the initiation of lipid peroxidation. For example, healthy volunteers who drank green tea (100 mg total catechins/day) for 4 weeks showed sparing of their plasma vitamin E and β-carotene levels.[137] Also, flavonoids may inhibit LDL oxidation by scavenging superoxide anions, hydroxyl radicals, or lipid peroxyl radicals. Alternatively, flavonoids may chemically modify LDL and such modification results in LDL being less susceptible to oxidation. Research continues into the various antioxidant modes of the polyphenols.[114] Since most polyphenol glycosides are water soluble, it is speculated that they should work in the aqueous phase of plasma and at the surface of lipoproteins. Vinson and Dabbagh[122] showed that catechins or green and black tea exhibited potent lipoprotein-bound antioxidant activity. However, van het Hof[86] observed that catechins were associated with HDLs, but the concentrations found in LDLs did not appear sufficient to enhance the resistance of LDLs to oxidation. In addition, it was proposed that resveratrol was associated with lipoproteins where it could scavenge oxygen free radicals.[138]

However, a number of *in vitro* studies have reported that wine, tea, or select polyphenols could inhibit LDL oxidation. Ishikawa and co-workers[139] observed that catechins could inhibit LDL oxidation in a dose-dependent manner *in vitro*, and EGCG appeared to be more potent than vitamin E. Interestingly, black tea theaflavins were more effective than catechins. Generally, studies have shown that wine polyphenols can inhibit oxidative changes of LDL, and red wine appeared more potent than white wine.[140,141] For example, the addition of 3.8 and 10 μM polyphenols extracted from red wine to LDLs *in vitro* inhibited its oxidation by 60 and 98%, respectively.[142] Red wine also inhibited cell-mediated LDL oxidation, while white wine and ethanol were not effective.[141] Red wine, catechin, or quercetin also produced inhibitory effects on development of aortic atherosclerotic lesions and reduced the susceptibility of LDL to aggregation[143] and subsequent atherogenic modification of LDL in atherosclerotic apolipoprotein E-deficient mice.[144] Polyphenols from grape extract also have the ability to inhibit oxidative changes of LDL.[145] However, although red wine and grape juice could inhibit LDL oxidation *in vitro*, LDL oxidation was only inhibited *in vivo* in those who drank wine.[146] Incubation of LDL with cupric chloride produced a lag phase of 130 min before the onset of a propagation phase. In the presence of grape extract the lag phase was extended 185, 250, and 465 min, respectively, when an 8000-fold, 4000-fold, and 2000-fold diluted grape extract was added to the LDL suspension. Frankel and colleagues[142] further observed that the inhibition of copper-catalyzed LDL oxidation by dilutions of wine could be mimicked by equal concentrations of quercetin, but inhibition by resveratrol was only about one half that of quercetin or epicatechin.[59]

White wines have expressed the ability to inhibit oxidation of LDL *in vitro*, but generally are not as effective as red wines.[140,141] About eight times more of white wine was required to produce

an effect equal to red wines on LDL oxidation and HDL concentration increases. However, if equal concentrations of wine polyphenols extracted from red or white wine were compared, inhibition of LDL oxidation was similar. Comparing dilutions of red or white wine, red grape juice, or beer, LDL oxidation was inhibited in a dose-dependent manner depending on the final polyphenol concentration.[147] Concentrations of ethanol ranging from 0.1 to 0.5% showed no inhibition, suggesting that it was the polyphenols in these beverages that was responsible for inhibiting LDL oxidation. Taken together, these studies suggest that the reported advantage of red wine over white wine in inhibiting LDL oxidation reflects the higher concentrations of polyphenols in red than white wine, rather than red wine polyphenols being more potent, on a mole-per-mole basis, than white wine polyphenols. However, the potencies of the various polyphenols acting alone vs. potential additive or synergistic effects of the combinations found in wines and tea have not been fully elucidated in the assay systems examined.

Several studies have estimated the relative potency of polyphenols compared with traditional antioxidants. The model often used is the *in vitro* oxidation of LDL suspension with cupric iron in the presence of different concentrations of polyphenols or antioxidants. Quality of the antioxidant is expressed as concentration of the substance to produce 50% inhibition of oxidation (IC_{50}). As listed in Table 14.4, individual polyphenols and the beverages containing mixtures of polyphenols have enormous potential to deter oxidative damage. Vitamin C, E, and β-carotenes are relatively weak antioxidants when compared with pure polyphenols or beverages containing polyphenols. Particularly strong antioxidant quality is expressed by the individual flavonoids quercetin and EGC in wine, tea, and grapes. It should be noted, however, that the greater ability of wine polyphenols to inhibit LDL oxidation *in vitro* than equal concentrations of vitamin E may simply reflect the limited availability of vitamin E to LDL when incubated *in vitro*.[148] Therefore, although the

TABLE 14.4
Antioxidant Quality of Selected Polyphenols and Antioxidants

Antioxidant/Polyphenol	Antioxidant Quality, IC_{50} (μM)
Vitamin C	1.25–1.45
Vitamin E	2.40
β-Carotene	4.30
Trolox	1.00
Quercetin	0.22
Rutin	0.41–0.51
Myricetin	0.48
Kaempferol	1.82
Catechin	0.42–0.67
Epigallocatechin (EGC)	0.075–0.10
Epicatechin	0.19–0.40
Resveratrol	0.33
Red wines[a]	0.72
White wines[a]	0.71
Grapes[a]	0.2–0.65
Black teas[a]	0.59–0.86
Oolong teas[a]	0.57–0.67
Green teas[a]	0.22–0.52

[a] Units of μmol/l for beverages.
Values based on 50% inhibition of LDL oxidation (IC_{50}) as described in text.

Modified from References 50, 132, and 186.

interpretation of the data presented in Table 14.4 may be difficult relative to vitamin E, the overall picture illustrates the potency of flavonoids. However, the data also indicate that care must be taken in reaching conclusions based solely on a single *in vitro* assay.

Despite the positive results suggesting that wine polyphenols can inhibit LDL oxidation *in vitro*, few studies have examined these effects *in vivo*. Hayek and colleagues[144] fed atherosclerotic apolipoprotein-E-deficient mice red wine, quercetin, or catechin in their drinking water for 42 days. They observed that LDLs isolated from these mice were more resistant to oxidation than LDLs isolated from ethanol-fed control mice. In addition, quercetin appeared to inhibit LDL oxidation more than catechin, whereas catechin appeared more effective in inhibiting LDL aggregation. When red or white wine polyphenols were fed to healthy men (1 g/day equivalent to 375 ml of wine for 2 weeks), the red wine group showed a greater increase in the LDL oxidation lag time than the white wine group.[126] Others have also demonstrated that red wine consumption by healthy volunteers reduce the susceptibility of their LDL to oxidative damage.[103,149] To date, the study by Fuhrman and colleagues[150] shows the most significant *in vivo* effects of red wine consumption on inhibiting LDL oxidation. They fed 17 healthy men 400 ml of red or white wine for 14 days. Plasma collected from red wine drinkers at the end of the study was about 20% less likely to peroxidize than plasma collected at baseline, and LDLs isolated from these subjects were more resistant to oxidation, effects independent of plasma vitamin E or β-carotene concentrations. Interestingly, they reported that plasma taken from white wine drinkers showed a 34% increase in lipid peroxidation compared with baseline and LDLs isolated from these subjects were more prone to oxidation. However, the study has come into question because the results obtained far exceeded the clinical benefit obtained previously by dietary or pharmacological interventions to prevent LDL oxidation.[151] Also, in 24 healthy subjects who drank 550 ml of red or white wine containing 3.5% ethanol for 28 days, no significant inhibition of copper-catalyzed LDL oxidation was observed.[151] Similar negative results were seen in 20 volunteers who drank 200 ml of red or white wine for 10 days.[105]

Consumption of tea or their polyphenols have also been reported to inhibit LDL oxidation. Green and black tea were observed to inhibit LDL oxidation in rabbits by 13 and 15%, respectively,[109] and LDL oxidation was inhibited when compared with controls in LDL-receptor deficient mice fed black tea for 8 weeks.[152] In 14 healthy volunteers, consumption of 750 ml of black tea for 4 weeks prolonged the lag phase for LDL oxidation from 54 to 62 min.[139]

However, it was also reported that flavonoids could accelerate LDL oxidation if they were added to minimally oxidized LDL.[153] In addition, some flavonoids enhanced LDL aggregation.[154] Since atherosclerotic plaque contains high concentrations of copper and iron,[155] the net *in vivo* effect of polyphenols on LDL oxidation cannot be predicted easily. Therefore, despite the *in vitro* inhibition of LDL oxidation and the acute rise in serum antioxidant potential following consumption of red wine or tea in particular, the limited human data do not provide strong evidence that a major *in vivo* effect of wine or tea polyphenols is inhibition of LDL oxidation. Further work is needed to define whether these results simply reflect insufficient absorption and/or deposition of the necessary polyphenols into the target tissues.

D. Nitric Oxide-Related Effects

Nitric oxide (EDRF:endothelial-derived relaxing factor), synthesized from arginine, is a major promoter of vascular relaxation that also inhibits platelet adherence to endothelium. Evidence suggests that wine polyphenols may modulate the production of nitric oxide (NO), since wines, grape juices, and extracts from grape skins relaxed precontracted rat aortic rings. In addition, quercetin and tannic acid could reproduce the effects of wine and grape fractions, whereas resveratrol, catechin, and malvidin could not.[156,157] On the other hand, 0.1 and 0.2 m*M* quercetin inhibited lipopolysaccharide-stimulated inducible nitric oxide synthase mRNA, the release of NO, and the production of TNF-α from a macrophage cell line.[158] In addition, some flavonoids, including anthocyanidins and catechin in pure forms, have been shown to exhibit NO-scavenging

properties,[118,159] which may account for the observation that catechin did not induce vasorelaxation.[160] Other data indicate that quercetin may act, at least in part, through an NO-independent mechanism. Nevertheless, it remains unknown whether these substances would have an effect on NO metabolism or vascular relaxation after oral consumption that could translate into reduced risk for CVD.

In addition, both red and white wine contain significant amounts of salicylic acid and its dihydroxybenzoic acid metabolites, which also have vasodilator and anti-inflammatory activities.[161] It was determined further that the concentrations of these compounds in wine range from 11 to 28.5 mg/l, with the concentrations being higher in red than in white wine. Again, it remains to be determined whether these compounds are absorbed after drinking wine or whether plasma concentrations attained would have the physiological effect observed *in vitro*.

E. Effects on Hemostasis

Wine may also have a positive influence on risk factors of CVD by inhibition of platelet aggregation and prolongation of clotting times. Ethanol has long been known to exert an aspirin-like effect on clotting mechanisms[21]; however, the concentrations required to inhibit platelet aggregation have generally been high.[22,162] In patients with coronary artery disease, those who drank 330 ml of beer per day for 30 days showed evidence of reduced thrombogenic acitivity compared with patients who did not consume an alcoholic beverage.[163]

Both intravenous or intragastic administration of red wine or grape juice, but not white wine, inhibited platelet-mediated thrombus formation acutely in stenosed coronary arteries in dogs.[162] In addition, low concentrations (n*M*) of quercetin dispersed platelet thrombi that adhered to rabbit aortic endothelium *in vitro*.[164] It was shown that platelet aggregation was inhibited about 70%, compared with controls, in rats fed ethanol, white wine, or red wine in their drinking water for 2 or 4 months.[165] However, if the ethanol or wine was withheld for 18 h, platelet aggregation rebounded to greater than control levels in the groups fed ethanol or white wine, whereas aggregation was still inhibited in the red wine group. In this study, the authors focused on the role of tannins (procyanidins) in red wine on inhibition of platelet aggregation.

A number of investigations have examined the effects of wine on hemostasis in humans. Lavy and colleagues[103] observed that in 20 healthy men who consumed 400 ml of red or white wine for 14 days prothrombin time increased and partial thromboplastin time decreased significantly in both groups. When collagen was employed as agonist, no significant change in platelet aggregation was observed following consumption of either wine. Also, no significant effects on platelet aggregation were observed in 20 subjects with hypercholesterolemia who drank red or white wine for 28 days.[128] In contrast, collagen-induced platelet aggregation was lower in male volunteers who consumed 30 g of ethanol/day (about two to three drinks) in the form of red wine or ethanol-spiked clear fruit juice for 28 days when compared with subjects who drank dealcoholized red wine.[166] However, no difference in platelet aggregation was observed if ADP was employed as agonist. These data prompted the authors to conclude that their observations were due to ethanol and not to components in red wine. On the other hand, Seigneur and colleagues[149] reported that ADP-induced platelet aggregation was inhibited in 16 subjects who drank red wine for 15 days. Epinephrine- or arachidonic acid–induced platelet aggregation was not affected in these subjects, nor was aggregation affected in subjects who drank white wine or an ethanol solution. A comprehensive study by Pace-Asciak and colleagues[167] had 24 healthy males consume 375 ml/day of red or white wine or grape juice without or with added *trans*-resveratrol (4 mg/l) with meals for 28 days. Only white wine inhibited ADP-induced platelet aggregation, whereas red and white wine and resveratrol-supplemented grape juice inhibited thrombin-induced platelet aggregation. *In vitro*, these authors observed that ADP- and thrombin-induced platelet aggregation was inhibited about 50% by grape juice without or with resveratrol, whereas red wine nearly abolished platelet aggregation and white wine had no appreciable effect.[167] In a previous *in vitro* study these authors reported that platelet

aggregation could be inhibited by dealcoholized red wine, quercetin, and resveratrol in a dose-dependent manner.[168] Again, these data suggest that the *in vitro* effects of wine and its polyphenols on platelet aggregation are more pronounced that the *in vivo* effects. Nevertheless, it remains to be determined whether these effects on platelet aggregation are of clinical importance and can translate into reduced risk from thrombi formation and the risk of serious stenosis or a coronary event.

Tea consumption has also been reported to reduce blood coagulation.[169] However, in another study, Vorster and colleagues[170] did not observe any significant effect of black tea on fibrinogen, tissue plasminogen activator, or plasminogen activator inhibitor-1.

VI. POTENTIAL ADVERSE EFFECTS OF POLYPHENOLS

Consumption of polyphenols through a variety of foods is not likely to produce adverse effects because of the diversity and varying quantities of polyphenols in plant sources. However, chronic pharmacological doses have been reported to produce adverse effects.[171] For example, doses of 1 to 1.5 g/day of cianidanol, a flavonoid drug, produced renal failure, hepatitis, fever, hemolytic anemia, thrombocytopenia, and skin disorders. In addition, hydrolyzable tannins in black teas are well known to inhibit iron absorption.[47,172] Green tea consumption has also been reported to reduce iron status, but neither black nor green tea significantly affected calcium, copper, zinc, or magnesium status.[173]

Sensitivity to EGCG reportedly has induced asthma in workers at a green tea factory.[85] In subjects consuming about 1 g/day EGCG supplements, approximately the dose found in people who drink >10 cups of green tea/day, some stomach discomfort was noted that resolved if the tablets were taken after a meal. Some transient sleeplessness was also reported, but could be due to caffeine contamination of the extract. The LD_{50} in rats is reported to be 5 g/kg in males and 3.1 g/kg in females, suggesting that EGCG has relatively low acute toxicity, but may express teratogencity at concentrations potentially achievable with daily consumption.

Quercetin also appears to be relatively nontoxic with an LD_{50} in mice over 100 mg/kg. In a Phase I clinical study, toxicity, expressed as nephrotoxicity, was not observed until a cumulative dose of 1700 mg/m^2 was achieved.[85] No evidence of carcinogenity or teratogenicity with quercetin has been reported, even when fed at dietary levels as high as 10%.[85]

A study in Portugal observed a dose-dependent relationship between red wine consumption and incidence of gastric cancer.[174] However, it is not known if this observation is related to the ethanol, red wine polyphenols, or their interaction with other risk factors. Free radicals have been identified in red wines and their originating grape source, but have not been detected in white wine.[175] Although their significance is not clear, phenolic compounds, including those found in red wine, have been shown to be mutagenic and genotoxic in bacterial and mammalian mutagenicity tests.[176,177] This may also be due to the concomitant production of hydrogen peroxide of phenolics during their autoxidation in a process that is dependent on divalent metal ions. This is similar to the metal-induced pro-oxidant effect of vitamin C involving the direct reduction of chelated Fe^{3+} to Fe^{2+} and succeeding radical generation. Flavonoids can also express pro-oxidant effects in the presence of Cu[178] or NO,[179] and Haliwell[148] reported that plant phenolics may show an oxidant effect against proteins and DNA. Conditions where phenolic compounds act as pro-oxidants have been described by Decker[180] and Laughton and colleagues.[181]

VII. CONCLUSIONS

A. Significance of Polyphenols in CVD and Health

The results from the studies summarized here indicate that the polyphenols present in grape wine and tea possess antioxidant activity, and have the potential to modify plasma cholesterol and lipoprotein concentrations, inhibit LDL oxidation, reduce platelet aggregation, and have

vasorelaxant effects, all of which have the potential to modify certain risk factors associated with the development of atherosclerosis or ischemic heart disease in susceptible individuals. The majority of these effects have been expressed *in vitro*, but the reproducibility of these effects *in vivo* has been less stellar. These differences may simply reflect low rates of absorption of the active compounds, differences in methodologies employed by the various investigators to detect these effects, or other factors. It is also realized that wines and teas are complex mixtures, and although techniques have improved to quantitate the various polyphenols in these drinks, investigators have attributed their observations to the substances they can measure, which may not be the most biologically active. Even in cases where specific polyphenols are studied, the results have not always been consistent. In studies where red wine is reportedly superior to white wine, the differences most likely merely reflect the higher polyphenol concentrations in red than white wine, rather than differences in the potencies of polyphenols that may be found in both wines. It is also generally agreed that the polyphenols in red wine are rather uniform and where different results are reported by investigators, they probably do not reflect the use of different varietal wines or wines from different regions. Since different processes are modified to produce the various teas in countries throughout the world, the uniformity of polyphenols in similar teas is unknown. In addition, although studies may attempt to control the amount of tea solids used, brewing times have not always been reported. It is reported that 84% of the antioxidant content of tea is extracted in the first 5 min of brewing, with an additional 13% extracted over the next 5 min.[121]

With respect to atherosclerosis and heart disease, in studies that have reported increased antioxidant potential in plasma after wine or tea consumption, the changes have been transient and disappear a few hours after drinking. In the platelet aggregation studies, results have been reported for only one or two time points after drinking, and aggregation may change in response to one agonist, but not another, making the overall physiological significance difficult to interpret. Thus, it remains to be established whether these results would be sustained after continued consumption. Insight into the long-term significance of these effects awaits comparing these variables in human populations who consume wine or tea frequently with populations where consumption of these beverages is less frequent or very little.

B. Dietary Recommendations

As mentioned above, polyphenols are also present in a number of common fruits and vegetables.[37,45,69] A number of studies have touted the potential health benefits of consuming diets rich in fruits and vegetables, e.g., protection from cancer. Since it is unclear which of the polyphenols offers the most health-promoting advantage, it would seem premature and inappropriate to recommend consuming wine or tea polyphenols specifically in an attempt to raise an individual's plasma antioxidant status as a means to reduce risk of CVD. Even in the case where wine may contain a particular polyphenol not generally found in other common foods, such as resveratrol, the evidence that it possesses any specific protective effects against CVD *in vivo* is insufficient. On the other hand, there is sufficient information to suggest that, for adults, consuming one to two glasses of wine with meals, or moderate amounts of decaffeinated tea, would not be harmful.[182] However, the best evidence to date suggests that it would require at least 8 to 10 cups of tea/day to achieve a significant change in plasma antioxidant status.[121] In considering any recommendation for wine, one must always keep in mind the well-known adverse health effects and consequences of chronic ethanol abuse or the effects and risks of acute inebriation.[11,183,184]

C. Directions for New Research

From the foregoing discussion, it would appear that despite a wealth of *in vitro* studies citing the potential efficacy of wine and tea polyphenols against known risk factors of CVD, much research

is required to confirm benefit *in vivo*. Many of these studies employed high doses of flavonoids and additional studies could address repeated administration of lower doses and/or identify definitively other polyphenols in wine, grapes, and tea that may be important to human health. It is also unknown whether individual polyphenols can act in an additive or synergistic fashion *in vivo*. Since some polyphenols appear to be mutually exclusive in nature,[114] it is not known whether packaging them together in a supplement would be potentially harmful. In addition, attempts to identify optimal intake for these non-nutritive compounds will require further studies of their bioavailability from beverages, foods, and dietary supplements currently being sold, as well as studies of their metabolism and development of bioassays to determine efficacy. Once efficacy is established, additional studies could attempt to increase the bioavailability of those compounds most relevant to human physiology and health.

D. Final Comments

In summary, the available data to date indicate that wine and tea polyphenols, as well as the complex beverage, possess biological activity that may potentially modify certain risk factors associated with atherogenesis and CVD. Indeed, these polyphenols have the potential to be an important source of non-nutritive antioxidants in the diet.[121] Epidemiological studies suggest that the population that may benefit most from these compounds is probably those 35 to 65 or 70 years of age. Unfortunately, the data are slim to suggest convincingly that these substances may offer long-term protection from these diseases. There is also no definitive evidence that wine or tea consumption would be beneficial in trying to overcome the adverse health consequences of consuming a diet high in saturated fat or smoking. Since the best polyphenols to promote health are not known, it is premature to recommend dietary supplements containing individual compounds or complexes. Currently, based on an individual's preference and available evidence, there is nothing to suggest that adding a glass of wine with the meal or drinking tea would be harmful. Both beverages should be enjoyed for themselves. As usual, eating or drinking "in moderation" should remain the best recommendation.

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15 Olive Oil and Health Benefits

Denis M. Medeiros

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I. INTRODUCTION

The olive is a common name for a plant family and its representative genus, and for the fruit of the olive tree. There are approximately 900 species of olives in 24 genera. Most are familiar with the olive that is cultivated for its fruit, which is sometimes referred to as drupes. Olives for eating are harvested or picked when they are either unripe or ripe. The unripe olives are green and remain so during pickling. Ripe olives are dark bluish when fresh and turn blackish during pickling.

Olives have been associated with Mediterranean cultures for some time. The cultivated olive is originally native to the eastern Mediterranean region but is cultivated throughout that area and in other parts of the world that have climates similar to the Mediterranean area. The genus and species of the cultivated olive is *Olea europea*, which is grown between the 30th and 45th parallels. Spain, Italy, and Greece are the major producers of olives. More countries and regions of the world (United States, Canada, Japan) are cultivating olives due to the interest in the health benefits of the Mediterranean diet.

The nutrient composition of olives is shown in Table 15.1. One large olive will supply 5.1 kcal. Most of the caloric value is supplied by fat, followed by carbohydrate and protein, respectively. Olive oil is derived from the fresh, ripe fruit and comprises about 20% of the olive by weight. One of the most studied aspect of olives is the fatty acid content, with the oil being a good source of the monounsaturated fatty acid oleate. Oleate may range from 56 to 84% of the fatty acid content.[1] Olive oil also contains the saturated fatty acids palmitoleate and stearate in small amounts, the polyunsaturated fatty acids linoleate, and, to a small degree, linolenate.[2] Linoleate may comprise 3 to 21% of the fatty acid content.[1]

The highest-quality olive oil is termed virgin oil. This is the oil that is first expressed under light pressure during processing and not further refined. Most olive oil on the market is expressed under heavy pressure and undergoes further refinement. Typically, olive oil may oxidize easily and produce a strong flavor. Thus, protection from light and heat will increase its shelf life considerably.[3]

The interest in the health benefits of olive oil is due to the low incidence of coronary heart disease and even cancer, particularly breast cancer, in cultures where a "Mediterranean diet" is consumed. This diet is rather high in fruit, vegetables, grains, and legumes, but low in meat. Much of the evidence that links the Mediterranean diet to a lower incidence of coronary heart

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TABLE 15.1
Selected Nutrient Compositions of Olives

Nutrient	One Large Olive (4.4 g)
Macronutrients	
Water	3.52 g
Energy	5.05 kcal
Protein	0.037 g
Total lipid	0.47 g
Carbohydrate	0.28 g
Total dietary fiber	0.14 g
Ash	0.10 g
Lipids	
Palmitic (16:0)	0.05 g
Stearic (18:0)	0.01 g
Oleic (18:1)	0.34 g
Linoleic (18:2)	0.04 g
Linolenic (18:3)	0.003 g

Note: See Reference 2.

disease has centered around the relatively high oleate, but low saturated fat content. Diets in the Mediterranean area are characterized by a high content of oleic acid compared with diets in other Northern European cultures and North America. It is well known that monounsaturated fatty acids may lower blood cholesterol levels and may increase high-density lipoprotein (HDL)-cholesterol levels, which may be a link between olive consumption and the lower incidence of coronary heart disease. With respect to olive oil intake and cancer rate, the mechanism for such observations is less clear.

This chapter focuses on two health conditions as affected by olive consumption: coronary heart disease (CHD) and cancer. The components of olives in addition to fatty acids will be evaluated, as well as other potential compounds such as the polyphenolic compounds.

II. CORONARY HEART DISEASE

A. Fatty Acids in the Mediterranean Diet

Prescription of a Mediterranean diet to patients who have had a myocardial infarction decreases the risk of a second cardiovascular accident.[4] The reason for this finding may be due to several factors. It is commonly accepted that saturated fatty acids are twice as effective at raising blood cholesterol as polyunsaturated and monounsaturated fatty acids are at lowering blood cholesterol. The general consensus appears to be that mono- and polyunsaturated fatty acids are similar in terms of their cholesterol-lowering abilities. Several studies have shown that monounsaturated fatty acids decrease total blood cholesterol, low-density lipoprotein (LDL)-cholesterol, apolipoprotein B, and triglycerides, with no changes in HDL-cholesterol and Apo-I plasma levels.[5] Elder and Kirchgessner[6] reported that rats fed linseed oil as opposed to olive oil had lower concentrations of cholesterol, triglycerides, and phospholipids in plasma and lipoproteins, but a higher susceptibility of LDL to lipid peroxidation. This latter factor, the susceptibility of LDL-cholesterol to oxidation, yields a more potent atherogenic compound and may be a more significant factor. Also, while polyunsaturated fatty acids may lower blood lipids,[7,8] they elevate the oxidative susceptibility of LDL in contrast to fats that contain elevated saturated and monounsaturated fatty acids.[9,10]

B. Other Olive Constituents and Their Effects

It is not always clear if the resistance to oxidation from consuming the Mediterranean diet is due only to oleic acid and/or to other nontriglyceride components present in oleic acid–rich oils. The minor constituents of virgin olive oil are nonglycerides such as hydrocarbons, monoglyceride esters, tocopherols, alkanols, flavenoids, anthocyanins, hydroxy and dihydroxyterpenic acids, sterols, polyphenols, and phosopholipids.[1,11,12] The Mediterranean diet is high in polyphenolic compounds, and olives have a high level of these substances. The level of these compounds is variable, with 50 to 800 mg/kg olive oil reported. The level of these compounds in olive oil is dependent upon several agronomic factors, including soil, degree of olive ripeness, and cultivar or olive variety.[1] There are a number of phenolic compounds in extra virgin oil (Table 15.2). The simple phenolic compounds are hydroxytyrosol (3,4-dihydroxyphenylethanol), tyrosol, and phenolic acids such as vanillic and caffeic acids. The complex phenolic compounds are tyrosol, hydroxytyrosol esters, oleuropein, and its aglycone. Oleuropein is the phenol that contributes primarily to the bitter taste of olives,[1,12] but other phenolic compounds may contribute some bitterness as well.

TABLE 15.2
Phenolic Compounds in Extra-Virgin Olive Oil

Hydroxytyrosol
Tyrosol
Oleuropein
Vanillic acid
Caffeic acid

Phenols are very good antioxidants. The greater the phenol content in virgin olive oil, the better the oxidative stability. Hyroxytyrosol can donate a hydrogen to free radicals, thereby neutralizing their potential harmful effects as demonstrated in Figure 15.1. Another factor is that hydroxytyrosol is able to chelate metal ions, which are themselves pro-oxidant agents. However, it is important that metal ions be removed during processing since their presence can lead to partial degradation of the phenolic compounds in the oil.[13]

With respect to the ability of the various phenolic compounds to protect against LDL-cholesterol oxidation, both hydroxytyrosol and oleuropein inhibit $CuSO_4$-induced oxidation of LDL, and the effect appears dose dependent. Both luteolin and lutean aglycon are effective in protecting against LDL oxidation.[14,15] Visioli and Galli[1] reported that oleuropein and hydroxytyrosol are either equally or more effective than other antioxidants such as butylated hydroxytoluene (BHT), vitamin C, and vitamin E.

The phenolic compound hydroxytyrosol, present in olives, is a diphenolic compound common in extra virgin olive oil and it may be a potent antioxidant. The superoxide radical (O_2^-) and nitric oxide (NO^-) react rapidly to form peroxynitrite ($ONOO^-$), which is a chemical that is very reactive and can cause tissue damage. Nitric oxide may contribute to inflammatory diseases and cardiovascular disease. Hydroxytyrosol has been shown to be highly protective against the peroxynitrite-dependent nitration of tyrosine and DNA damage by peroxynitrite *in vitro*.[16] On the other hand, oleuropein can increase nitric oxide release by cultured macrophages after endotoxin challenge by increasing nitric oxide synthase expression. This may be beneficial in the sense that nitric oxide may gaurd against infectious agents and parasites.[17]

Incubation of LDL with olive oil phenolics (oleuropein or hydroxytyrosol) reduced the fall in vitamin E levels. Normally, virtually all of the vitamin E would have disappeared in 30 min, but 80% remained in the presence of phenols. Lesser amounts of compounds, such as isoprostanes, malonaldehyde, and lipid peroxides, were present. The presence of these substances is relatively

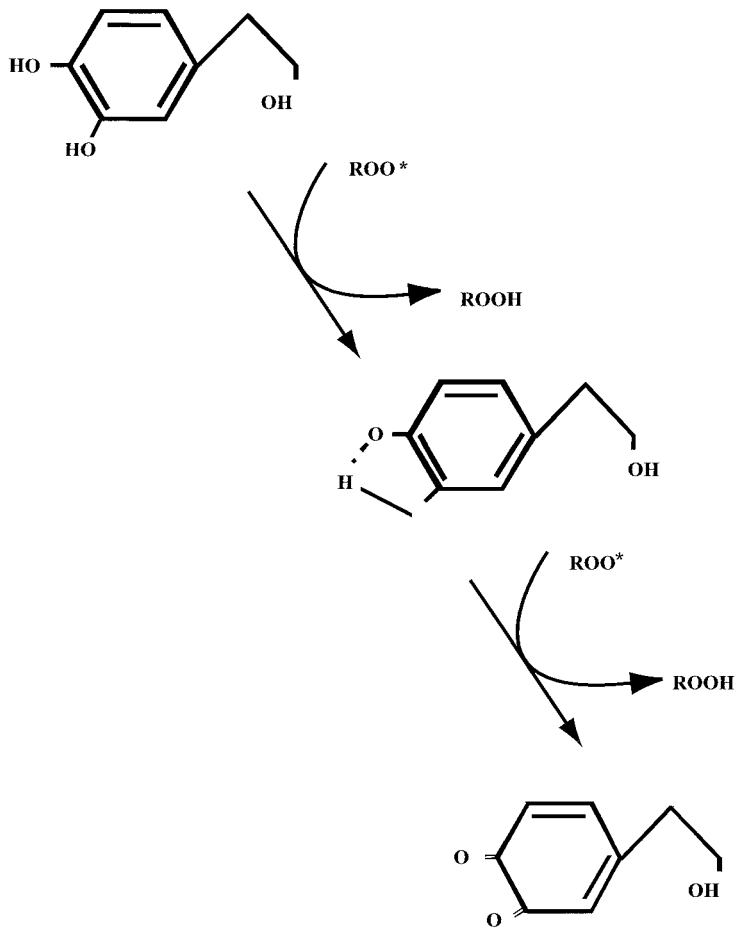

FIGURE 15.1 Antioxidant mechanism of hydroxytyrosol. Hydrogen is donated from the hydroxyl groups of the phenol ring structure to free radicals to generate stable compounds. Two hydrogen atoms per compound can react, resulting in a carbonyl structure on the phenol ring of the hydroxytyrosol.

indicative of free radical activity. Also, both phenolic compounds prevented the oxidation of linoleic and docosahexaenoic compounds in the LDL phosopholipids. Phenols can also inhibit platelet aggregation. Reduced TXB_2 and LTB_4 production by activated leukocytes is a known effect of olive phenolics.[1,12] In one study, Nicolaiew and colleagues[11] used ten normolipidemic subjects in a crossover design in which each received virgin olive oil or sunflower oil for 3 weeks each.[11] Plasma levels did not change after feeding of both diets in either the fasting or postprandial states. LDL oxidation, as measured by the formation of conjugated dienes, decreased after the olive oil diet. The results were mixed, in that there was a decrease in the level of conjugated dienes at the beginning and at the end of the oxidation reaction, but the total diene production (maximal – diene at time zero) in the presence of $CuSO_4$ did not differ.

III. CANCER AND OLIVE OIL

In modern cultures, a switch from a low-fat diet that contains a high proportion of monounsaturated fatty acids to a high-fat diet containing a high proportion of saturated fatty acids may have contributed to an increased incidence of cancer, including breast cancer. There is geographic variation in the incidence of breast cancer, and this variation is coincident with the consumption

of a high oleic acid intake derived from olive oil typical of the Mediterranean diet.[18] Case-control studies have yielded evidence of a protective association between oleic acid or olive oil consumption and breast cancer. Animal experiments indicate that oleic acid may be protective when ingested in a vehicle both very high in oleic acid and very low in linoleic acid, which is typical of olive oil. Consumption of olive oil has been shown to reduce mammary tumor incidence even in comparison with safflower oil containing similar amounts of oleic acid but higher levels of linoleic acid.[19,20] Experiments using rats and feeding a 15% olive oil diet significantly reduced tumor incidence caused by the carcinogenic compound 9,10-dimethyl-1,2-benzanthracene.[21]

Simsonsen and colleagues[22] hypothesized that an olive oil diet could reduce susceptibility of tissue structures to damage by free radicals, and thus the incidence of breast cancer. This research group used gluteal fat aspirates and measured the fatty acid profiles of subjects from various European cultures. Oleic acid showed a strong inverse relation with breast cancer in the Spanish cultures, but not among subjects from Berlin, Northern Ireland, the Netherlands, and Switzerland. In all, 291 patients with postmenopausal-incident breast cancer and 351 control subjects were studied. Non-Spanish residents did not show such an inverse relationship. One reason for the failure of this study to show any relationship of oleic acid levels to breast cancer in the non-Spanish population could be that olive oil contains other compounds, such as the phenols and flavenoids, that are good antioxidants. That the Spanish residents obtained their oleic acid from olive oil and the other residents from other sources is a likely explanation for these results.

Epidemiological studies have yielded consistent results on the association of monounsaturated fatty acids or olive oil consumption and the incidence of breast cancer. Landa and colleagues[23] studied 100 breast cancer subjects and 100 controls using, again, a food frequency instrument. Those with breast cancer reported lower intakes of fish and fruits and vegetables compared with controls. Those with breast cancer also had lower intake of vitamin C and monounsaturated fatty acids. Martin-Moreno and colleagues[24] used a case-control study in Spain and examined specific nutrient intakes using a food frequency questionnaire in 762 women with newly diagnosed breast cancer and compared this with 988 women randomly selected as controls. Total fat and type of fat intake were not associated with breast cancer in either pre- or postmenopausal women, after adjustment for energy intake. However, a lower risk of breast cancer was reported in those with a higher consumption of olive oil. Trichopoulou and colleagues[25] used a semiquantitative food frequency instrument administered to 820 women with breast cancer and 1548 control women from Greece to estimate the intakes of olive oil, margarine, and other food items. After adjustment for some other potential confounding factors, increased olive oil consumption was associated with a significantly reduced risk for breast cancer. Margarine consumption was associated with a greater risk of breast cancer. They also reported that fruit and vegetable consumption was inversely related to breast cancer in the same study. In a much larger study in Italy, 2564 women hospitalized with breast cancer were compared with 2588 women admitted to the same hospital for other health conditions not related to breast cancer, hormone problems, or gastric disorders.[26] Using a food frequency questionnaire, this study demonstrated an inverse relationship with olive oil and other vegetable oil consumption and the incidence of breast cancer. No relationship for butter or margarine was reported.

In addition to the role of olive oil in lowering the incidence of breast tumors, later studies suggest that other cancer types may benefit from a diet high in olive oil. Franceschi and colleagues[27] examined cases of 512 men and 86 women from northeastern Italy who had cancer of the oral cavity and pharynx and compared them with 1008 men and 483 women controls that had been admitted to area hospitals for ailments other than neoplastic conditions. Subjects were administered a dietary questionnaire to evaluate fat intake and other lifestyle aspects. Risk for these cancers were reduced by at least 50% in subjects with the highest intakes of several food items, including poultry, fish, raw and cooked vegetables, citrus fruit, and olive oil.

Although much of the work on olive oil intake and cancer has focused upon the monounsaturated fatty acid content, the antioxidant compounds present as reviewed above may also play an important

role, as they apparently do for heart disease. The studies examining the antioxidant effects of olive oil on various cancers are surprisingly limited and afford more opportunity for investigation.

IV. SUMMARY

Clearly, the monounsaturated content of the Mediterranean diet, via the intake of olive oil, plays a significant role in the lower incidence of both coronary heart disease and cancer, particularly breast cancer. The antioxidant compounds present in extra-virgin olive oil allow for the protection against LDL-cholesterol oxidation and also spare other antioxidant nutrients. The role of these same antioxidants in protecting against various cancers should be pursued. An examination of olive oil intake and other cancer types may also yield beneficial information.

The health benefits of olive oil that result from its active compounds should focus more on those agronomic factors that optimize their content. Additionally, further knowledge on the genetic regulation of the production of antioxidant phenolic compounds would be worthwhile. Increasing the content of these valuable nutrients to protect against both coronary heart disease and cancers is a good example of a functional food for health. Furthermore, extraction of these compounds from olives and concentrating them for clinical trials, both animal and human, may provide better insight into their utility as nutraceuticals for the future.

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16 Anticancer and Cholesterol-Lowering Activities of Tocotrienols

Najla Guthrie and Elzbieta M. Kurowska

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I. INTRODUCTION

Tocotrienols are a group of minor dietary constituents with potential therapeutic effects. Tocotrienols are naturally occurring analogues of tocopherol (vitamin E) found mainly in palm oil and cereal grains. They differ from tocopherols by possessing double bonds in the phytyl side chain. The four natural tocotrienol isomers, α, β, γ, and δ, differ in the number of methyl groups on the chromanol ring (Figure 16.1).[1] Tocotrienol-rich fractions (TRF) from palm oil usually contain tocotrienols α, γ, and δ, as well as 15 to 40% of α-tocopherol.

Dietary intake of tocotrienols is relatively high in many Asian countries traditionally using palm oil for cooking, but low in the rest of the world. Tocotrienols are well absorbed by the intestine, but the relationship between their absorption, blood levels, and tissue distribution is not fully understood. The pattern of disappearance from the circulation and tissue distribution is different for tocotrienols than for α-tocopherol.[2-3] Differences are also observed in tissue accumulation of tocotrienol isomers, suggesting their variable cellular uptake.[4]

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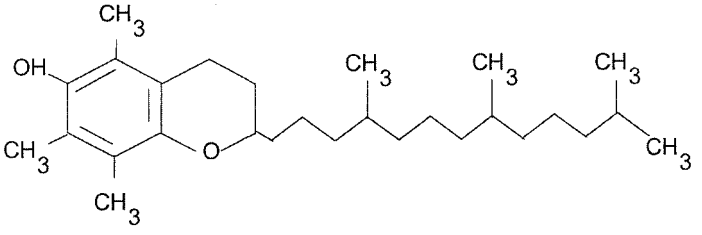
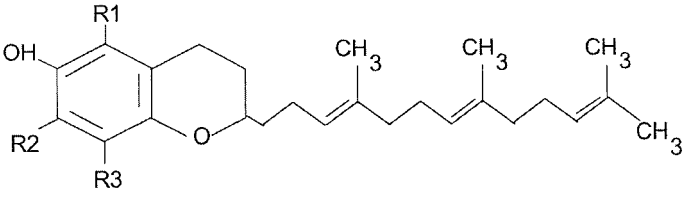
α-TOCOPHEROL



TOCOTRIENOLS



	R1	R2	R3
α-tocotrienol	CH_3	CH_3	CH_3
γ-tocotrienol	H	CH_3	CH_3
δ-tocotrienol	H	H	CH_3

FIGURE 16.1 Formulae of tocotrienols.

Dietary tocotrienol-induced health benefits are different from those induced by tocopherols. Tocotrienols appear to possess higher antioxidant activity than α-tocopherol, most likely due to the presence of double bonds in the side chain.[5,6] Furthermore, unlike α-tocopherol, tocotrienols have been reported to cause regression of atherosclerosis in some patients with cerebrovascular disease.[5] This chapter addresses the main therapeutic effects of tocotrienols, and anticancer and cholesterol-lowering activity *in vitro* and *in vivo*.

II. TOCOTRIENOLS AND CANCER

Tumors are cell populations with uncontrolled and abnormal growth and are classified as benign or malignant. Benign tumors do not invade the surrounding tissue. Malignant tumors aggressively invade surrounding tissues, altering their natural function. When malignant tumor cells spread to the lymph and circulatory systems, the metastatic cascade begins, spreading cancer cells throughout the body.[7] Control of cancer may be accomplished with suppressing, blocking, or transforming agents.[7] Suppression agents prevent the formation of other compounds capable of promoting new cancer formation from procarcinogens. Blocking agents prevent carcinogenic compounds from reaching the critical components into less toxic materials or prevent expression of the carcinogen. A variety of minor dietary components have been reported to possess anticancer activity. Among these compounds, tocotrienols have been shown by a number of different laboratories to have anticancer activity. The authors' interest in the anticancer properties of tocotrienols was stimulated by the results of studies with experimental animals and with cancer cells *in vitro*, as described below.

A. Studies with Experimental Animals

Unlike many other fats and oils, palm oil does not enhance the yield of chemically induced mammary tumors when fed to rats at high levels in a semipurified diet.[8] Evidence that this is related to the vitamin E fraction of palm oil was provided by Nesaretnam et al.[9] They showed that rats treated with 7,12-dimethylbenz(a)-anthracene (DMBA) to induce mammary tumors and fed vitamin E–free palm oil developed more tumors than those fed palm oil containing vitamin E. In a further study, they added the vitamin E fraction from palm oil to a 20% corn oil diet at either 500 or 1000 ppm. The rats receiving the higher level had a significantly greater median latency period, as well as a lower incidence and yield of mammary tumors than those fed corn oil alone or with the lower level of the vitamin E fraction.[9]

Other evidence that tocotrienols inhibit cancer has come from experiments with transplantable tumors in mice.[10,11] When injected intraperitoneally into mice, α- and γ-tocotrienols prolonged the life of mice bearing Ehrlich carcinoma, sarcoma 180, or implanted murine carcinoma (IMC). Gamma tocotrienol also prolonged the life of mice bearing Meth-A fibrosarcoma, but the compounds were ineffective against P388 leukemia. Gamma tocotrienol was more effective than α-tocotrienol in each case and α-tocopherol was least effective. These authors suggest that tocotrienols might exert antitumor activity by direct cytotoxic activity or by stimulation of the host immune system.[10] α-Tocotrienol and tocopherol were also reported to inhibit slightly the development of skin papillomas induced in mice by DMBA and promoted by tetradecanoylphorbol acetate (TPA).[11] Studies on the anticancer activity of tocotrienols have been reviewed by Elson.[12]

B. Experiments with Cells in Culture

1. Effects of Tocotrienols Alone and with Tamoxifen on Estrogen Receptor-Negative Cells

The effect of the TRF, α-, δ-, and γ-tocotrienols and α-tocopherol on MDA-MB-435 estrogen receptor-negative human breast cancer cells was investigated. The cells were incubated in the presence of varying concentrations of the compounds, and the incorporation of [^{3}H] thymidine into the DNA of the cells was measured. TRF inhibited the proliferation of these cells much more effectively than α-tocopherol.[13] When the tocotrienols were tested individually, they were found to be more effective than might have been expected from the results with TRF (Figure 16.2). γ-tocotrienol was the most effective in inhibiting these cells having an IC_{50} (concentration required to inhibit cell proliferation by 50%) of 30 μg/ml (Figure 16.2).[14] In other experiments, it was shown that the tocotrienols act synergistically with tamoxifen, an estrogen antagonist, used extensively in the hormonal therapy of breast cancer.[15] It acts mainly by blocking the stimulatory action of estrogens in hormone-responsive breast cancer. It is thus much more effective in inhibiting estrogen receptor-positive cancer cells, although it also has an effect on estrogen receptor-negative cells. γ-Tocotrienol inhibited these cells better than tamoxifen (see Figure 16.2).[14] When TRF, α-, δ-, or γ-tocotrienols were tested in 1:1 combination with tamoxifen, the combinations inhibited cell proliferative rate much more effectively than the compounds alone (Figure 16.3).[14]

2. Effects of Tocotrienols Alone and with Tamoxifen on Estrogen Receptor-Positive Cells

In subsequent experiments in our laboratory we tested the ability of TRF, the individual tocotrienols, and α-tocopherol to inhibit the proliferation of MCF-7 estrogen receptor-positive human breast cancer cells. TRF and the tocotrienols inhibited these cells more effectively than α-tocopherol, but not as effectively as tamoxifen (Figure 16.4).[14] The tocotrienols had much lower IC_{50} values in MCF-7 cells than in MDA-MB-435 cells with γ- and δ-tocotrienols being the most potent. When these compounds were tested in 1:1 combination with tamoxifen, the IC_{50} values were in most

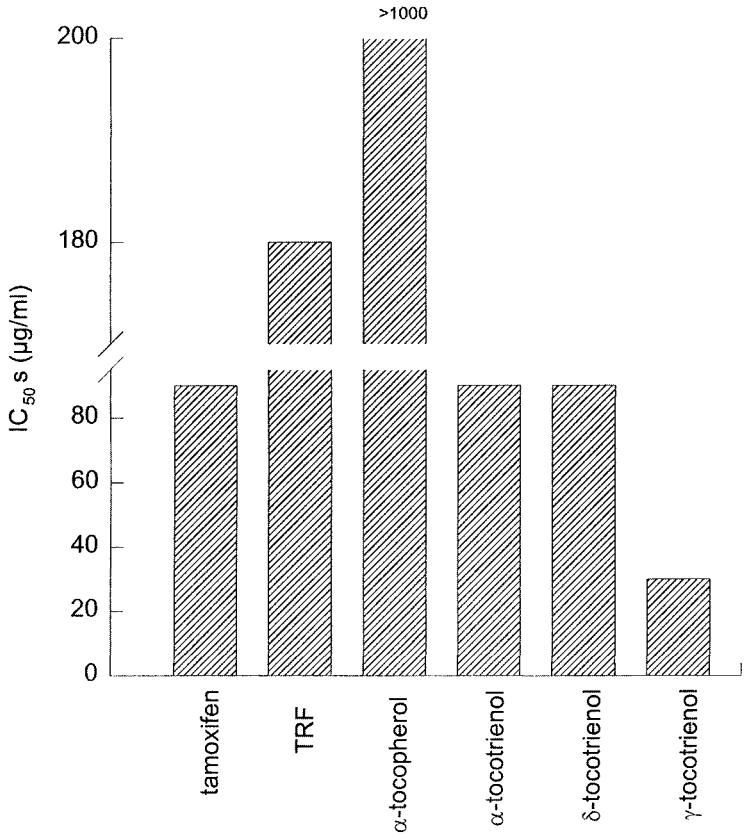


FIGURE 16.2 Inhibition of proliferation of estrogen receptor-negative cells by tocotrienols and tamoxifen.

cases not lower than that of tamoxifen alone. Only γ- and δ-tocotrienols in combination with tamoxifen gave lower IC_{50} values than either of the compounds alone (Figure 16.5).[14] In the treatment of cancer, combinations of drugs are often more effective than the individual drugs by themselves. The results of our *in vitro* experiments indicate that the same principle applies to anticancer compounds present in food.

C. Studies of the Mechanism of Action

1. Effects on the Estrogen Receptor

The effect of tocotrienols on the estrogen receptor was investigated. MCF-7 cells were depleted of steroids and treated with tocotrienols or tamoxifen (used as a positive control) in the presence or absence of 100 n*M* estradiol. The addition of excess estrogen did not reverse the inhibition by tocotrienols, whereas it did in the case of tamoxifen.[14] These results indicated that tocotrienols are not exerting their antiproliferative effects by acting as antiestrogens.

2. Effects on Protein Kinase C

Cellular mechanisms underlying the growth inhibition of a variety of cancerous cell lines by tocotrienols are still unclear, but are thought to be linked to inhibition of enzymes involved in transduction of mitogenic signals. Protein kinase C (PKC) is a component of one of the major signal transduction systems in eukaryotic cells.[16,17] Activation of the system leads to translocation

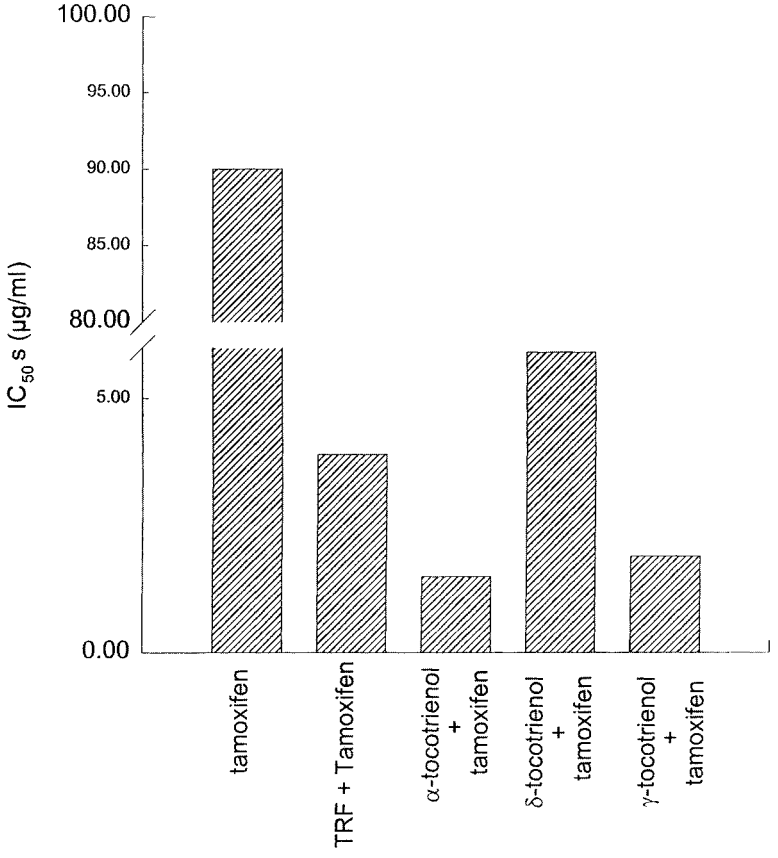


FIGURE 16.3 Inhibition of proliferation of estrogen receptor-negative cells by 1:1 combinations of tocotrienols and tamoxifen.

of PKC from the cytosol to the cell membrane where, in its activated form, it can phosphorylate serine and threonine residues of a variety of substrates, including cell surface receptors for growth hormones, such as epidermal growth factor receptor.[18] PKC is thought to play an important role in signal transduction that influences cell growth and transformation.[19,20]

Our studies on the effects of TRF, α-tocopherol, and the individual tocotrienols showed that these compounds were better inhibitors of PKC activity than that of proliferation as shown by the IC_{50} values for both (Figure 16.6).[21] In fact, TRF and the individual tocotrienols completely abolished PKC activity at the level of their IC_{50} for cellular proliferation of the cells.[21] This inhibition occurs in the membrane associated fraction.[21] Tamoxifen has also been observed to inhibit PKC in estrogen receptor-negative human breast cancer cells.[22]

3. Effects of Tocotrienols on Farnesyl:Protein Transferase and Geranylgeranyl:Protein Transferase Activities

Prenylation of proteins occurs by the enzyme-catalyzed transfer of farnesyl and geranylgeranyl moieties from the corresponding pyrophosphates to the proteins.[23] The prenyl group helps to anchor these proteins to cellular membranes. Some of these proteins, such as ras protein, are biologically active only when they are attached to the inner surface of the plasma membrane.[24] Much attention has been focused on ras proteins because of their involvement in many types of cancer. Several reports suggest that both membrane association and biological activity of ras proteins are abolished

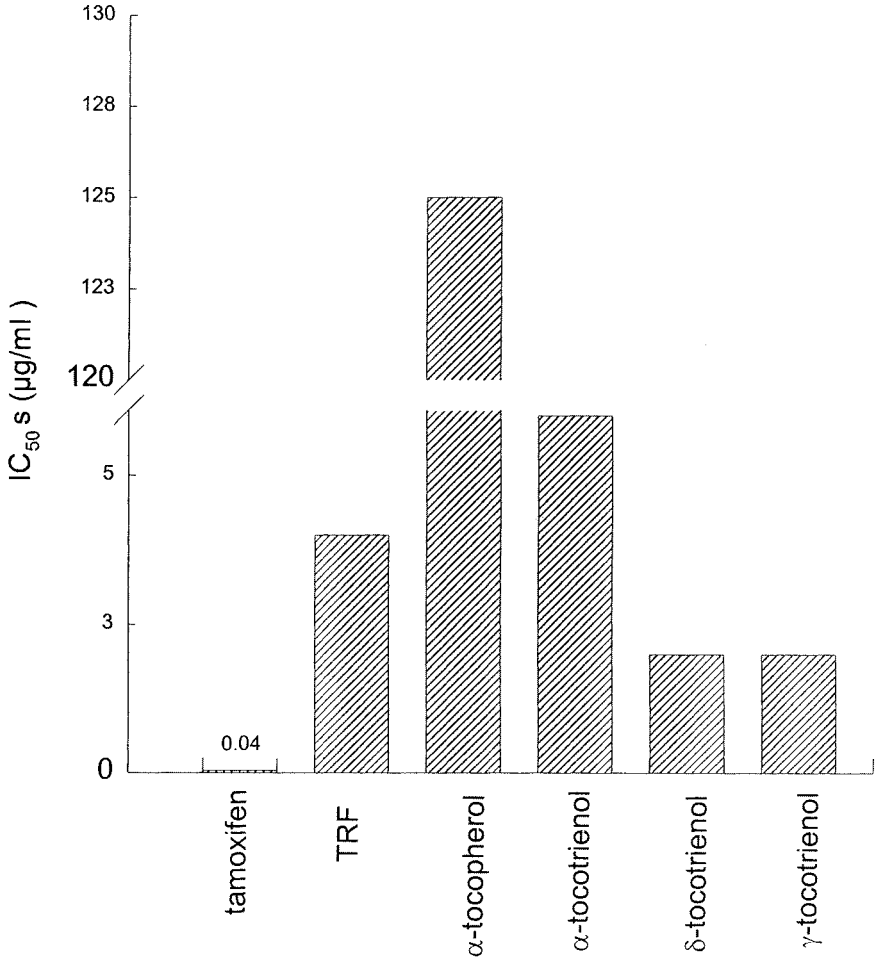


FIGURE 16.4 Inhibition of proliferation of estrogen receptor-positive cells by tocotrienols and tamoxifen.

by blocking farnesylation of these proteins.[25] Compounds that specifically inhibit farnesylation may thus be useful in controlling malignancies.[26]

Therefore, we investigated the ability of tocotrienols to inhibit the farnesyl:protein transferase and geranylgeranyl:protein transferase activities.[21] For these studies, PAP2 (ras-transformed NIH 3T3 mouse fibroblasts) cells, kindly provided by Dr. A.F. Chambers, were cultured and used as an enzyme source. Tocotrienols inhibited the proliferation of these cells, with δ-tocotrienol the most effective (Figure 16.7).[21] Our results showed that TRF and the individual tocotrienols inhibited farnesyl:protein transferase and geranylgeranyl:protein transferase more effectively than α-tocopherol (see Figure 16.7).[21] δ-Tocotrienol was the most effective in inhibiting farnesyl:protein transferase activity (see Figure 16.7).[21]

III. TOCOTRIENOLS AND HYPERCHOLESTEROLEMIA

Hypercholesterolemia associated with an elevation of low-density lipoprotein (LDL) cholesterol is a well-recognized risk factor for coronary heart disease (CHD), the leading cause of death in North America. The combination of high levels of LDL-cholesterol and oxidative stress contribute to the formation and development of atherosclerotic plaque, the underlying cause of CHD. Intake of antioxidant vitamins, such as vitamin E, has been suggested to lower the risk of CHD by

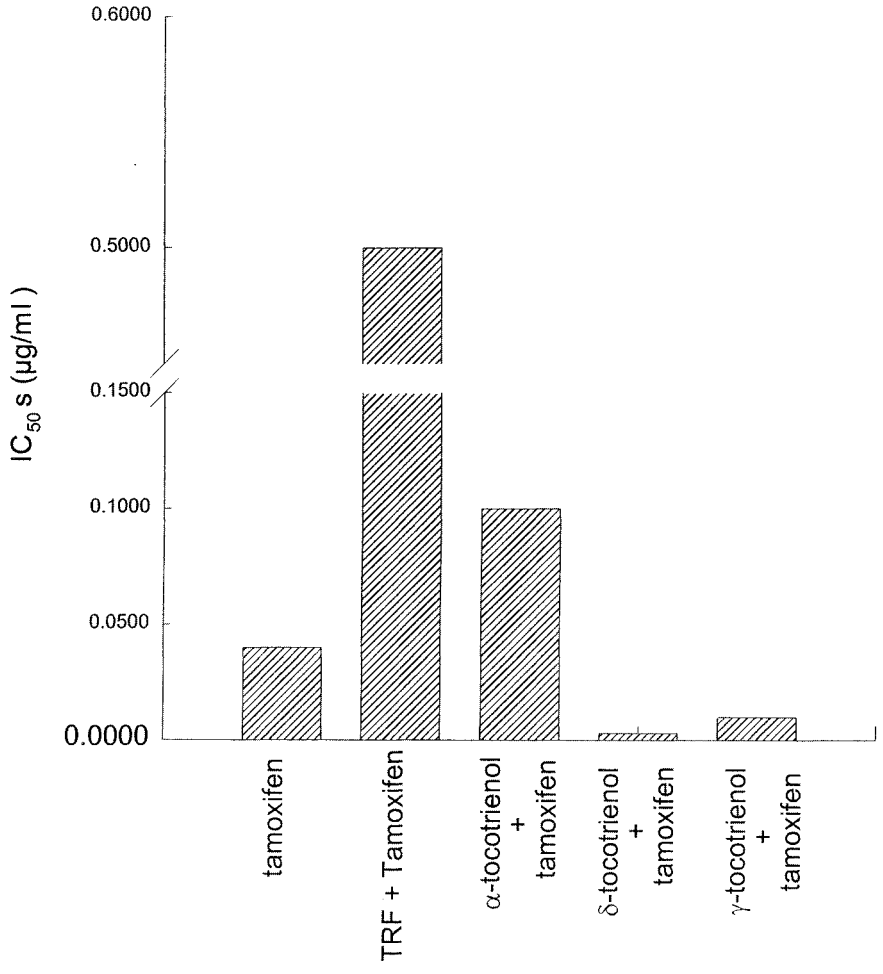


FIGURE 16.5 Inhibition of proliferation of estrogen receptor-positive cells by 1:1 combinations of tocotrienols and tamoxifen.

counteracting LDL oxidation.[27] Unlike vitamin E, tocotrienols appear to possess both LDL antioxidant and hypolipidemic properties, a profile that may be particularly antiatherogenic.[27] Better understanding of the mechanisms by which these compounds exert their cholesterol-lowering action should help develop new strategies useful in prevention of CHD.

Dietary supplementation with TRF and its constituent tocotrienols has been shown to produce cholesterol-lowering effects in several animal models.[28,29] Tocotrienol-rich preparations from palm oil or rice bran oil were also effective in reducing elevated plasma levels of total and LDL cholesterol as well as plasma apoliprotein B (apo B) concentrations in humans, although these responses were not reported in all clinical trials.[30–33] The beneficial effects of TRF preparations were postulated to be due to tocotrienols rather than α-tocopherol since, in earlier animal and human studies, dietary α-tocopherol alone produced little or no change in hypercholesterolemia.[1,28,29] However, some previous reports demonstrated that the cholesterol-lowering potential of tocotrienols could be modulated by their content of α-tocopherol.[34]

The ability of tocotrienols to reduce serum total and lipoprotein cholesterol and apo B levels has been attributed mainly to their cholesterol synthesis suppressive activity. Tocotrienols have been shown to inhibit the activity of HMGCoA reductase, the rate-limiting enzyme of hepatic cholesterol biosynthesis in human hepatoma HepG2 cells and in rat hepatocytes,[35] and this was

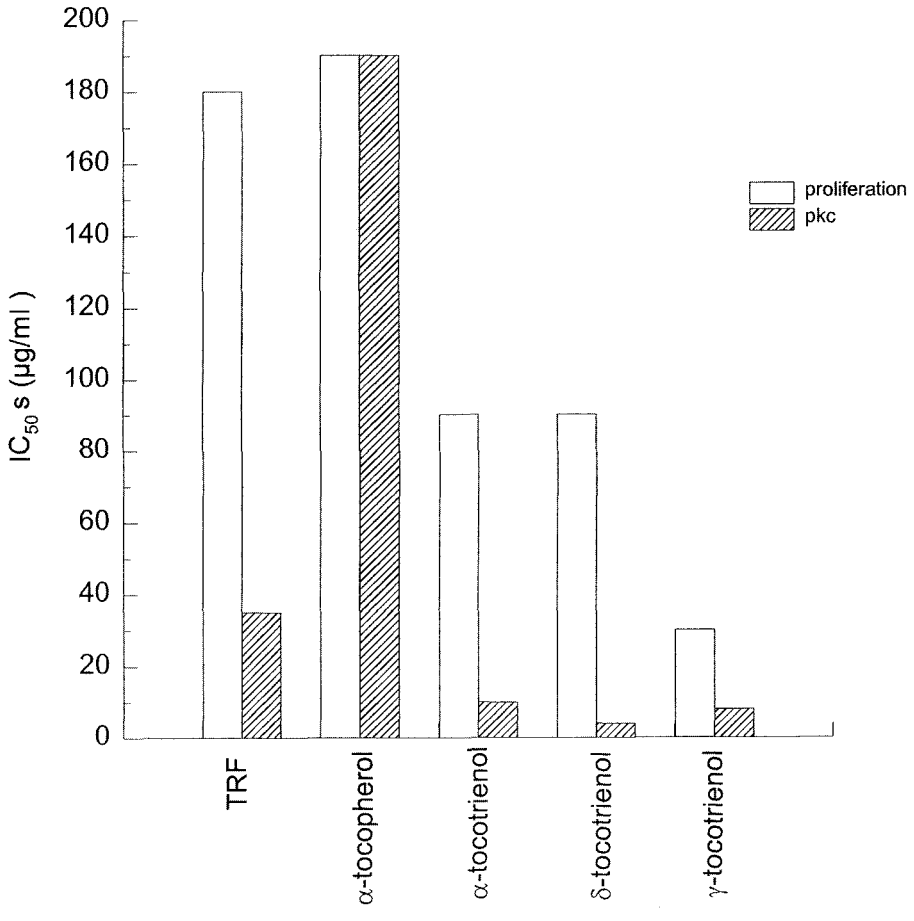


FIGURE 16.6 Inhibition of PKC activity by tocotrienols.

also confirmed *in vivo*.[1,34,36] In addition, tocotrienols have been suggested to modulate lipoprotein metabolism by other mechanisms. Previous studies demonstrated that, in HepG2 cells, γ-tocotrienol upregulated LDL receptor responsible for uptake and degradation of apo B–containing lipoproteins.[35] Also, in a recent report, γ-tocotrienol moderately reduced HepG2 cell production of apo B by increasing its intracellular degradation prior to secretion.[37] This last effect has been associated with a substantially reduced synthesis of intracellular cholesteryl esters (as well as free cholesterol) suggesting that γ-tocotrienol-induced changes in apo B metabolism were likely due to a decreased availability of lipids, especially cholesteryl esters, for assembly and secretion of apo B–containing lipoproteins.

The cholesterol-lowering potential of tocotrienol isomers and their mixtures was evaluated previously but only with respect to their cholesterol synthesis-suppressive activity. These experiments revealed that in HepG2 cells and in rat hepatocytes, both γ- and δ-tocotrienols were equally active as inhibitors of HMGCoA reductase whereas α-tocotrienol was less potent.[1] In rat hepatocytes, a combination of γ- and δ-tocotrienol also produced a greater inhibition of HMGCoA reductase than each isomer separately, possibly due to a synergy.[1] Recent results obtained for γ-tocotrienol[37] have suggested that the overall cholesterol-lowering potential of all tocotrienol isomers and their mixtures might partly depend on mechanisms other than the inhibition of HMGCoA reductase. These data indicated therefore that the overall cholesterol-lowering effect of tocotrienols and their mixtures should be evaluated by measuring their ability to reduce net production of apo B.

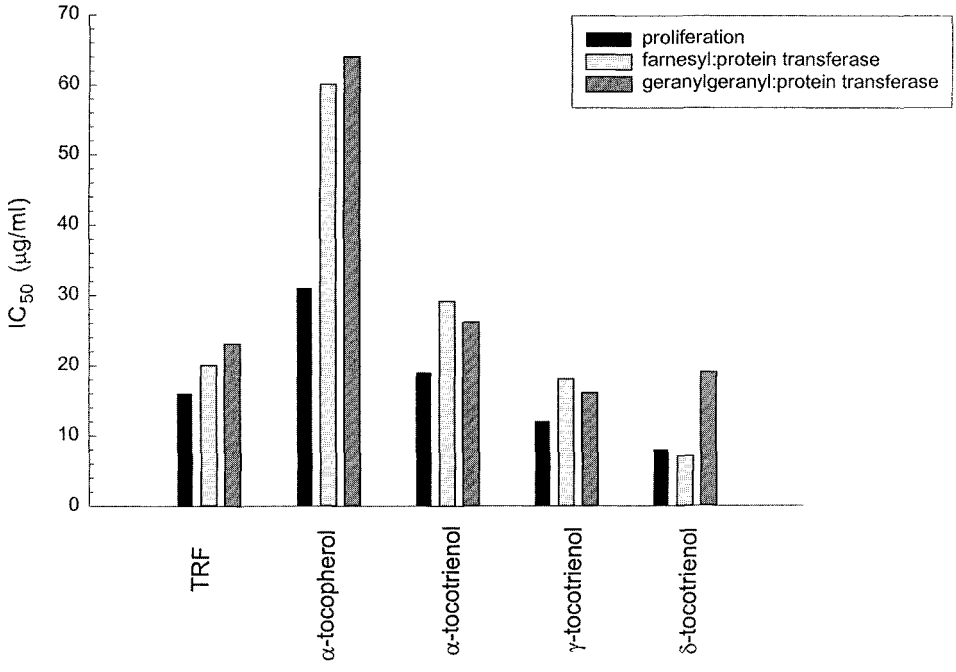


FIGURE 16.7 Inhibition of proliferation, farnseyl:protein transferase, and geranylgeranyl:protein transferase activities in PAP2 cells by tocotrienols.

A. Cholesterol-Lowering Responses of Individual Tocotrienols in Cells

The apo B-lowering potential of tocotrienols and their combinations, with and without α-tocopherol, was evaluated in HepG2 cells, which secrete and catabolize lipoproteins similar to LDL. Confluent cells were preincubated for 24 h in serum-free medium, which inhibits cell proliferation and stimulates biosynthesis of LDL-like lipoproteins. They were subsequently incubated for another 24 h in the same medium in the presence or absence of tocotrienols or α-tocopherol at the range of nontoxic concentrations. For each compound, the highest nontoxic concentration was predetermined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) viability assay.[38] At the end of the incubation, changes in medium concentration of apo B were evaluated by ELISA and compared with changes induced in the absence of vitamin E or its analogues using the methodology described previously.[39] As shown in Table 16.1, after 24-h incubation, all individual tocotrienols caused a dose-dependent reduction of medium apo B, whereas α-tocopherol had no

TABLE 16.1
Effects of Various Nontoxic Concentrations of Tocotrienol Isomers and α-Tocopherol on apo B Accumulation in the Media of HepG2 Cells

Concentration (μg/ml)	Percent apo B in Cell Culture Media			
	α-Tocotrienol	γ-Tocotrienol	δ-Tocotrienol	α-Tocopherol
0.00	100 ± 9	100 ± 14	100 ± 8	100 ± 17
6.25	78 ± 8	79 ± 2	69 ± 5	99 ± 8
12.50	69± 4	68 ± 3	49 ± 10	104 ± 7
20.00	46 ± 2	51 ± 7	10 ± 2	105 ± 5
50.00	35 ± 2			96 ± 6

effect.[40] The most pronounced 90% medium apo B reduction was induced in HepG2 cells incubated with δ-tocotrienol at a concentration of 20 μg/ml. A less substantial effect was observed in cells exposed to α-tocotrienol at a concentration of 50 μg/ml and in those incubated with γ-tocotrienol (65 and 49% medium apo B reduction, respectively). A comparison of IC_{50} concentrations (concentrations required to reduce medium apo B levels by 50%) revealed that δ-tocotrienol had greater ability to reduce medium apo B than α- or γ-tocotrienol (8.8 vs. 18.0 and 21.0 μg/ml, respectively). Since, as inhibitors of cholesterol biosynthesis, both γ- and δ-tocotrienols were previously reported to be equally potent and more active than α-tocotrienol, these results suggested that the apo B–lowering activity of all tocotrienols could partly depend on their ability to modulate cholesterol apo B metabolism via mechanisms other than the suppression of HMGCoA reductase.

B. Cholesterol-Lowering Responses of Tocotrienol Combinations in Cells

In the subsequent experiment designed to establish whether the apo B–lowering potential of tocotrienols could be altered by other tocotrienols or α-tocopherol, HepG2 cells were incubated for 24 h with these compounds (each at the concentration of 20 μg/ml) added in binary or triple combinations.[40] The results showed that when two or three tocotrienols were present together in cell culture medium, their apo B–lowering potential was enhanced due to either additive or synergistic effect (Figure 16.8). In contrast, addition of α-tocopherol generally impaired the apo B–lowering activity of individual tocotrienols or their mixtures, in agreement with previous results *in vivo*.[34] The interactions observed between tocotrienol isomers are consistent with the hypothesis

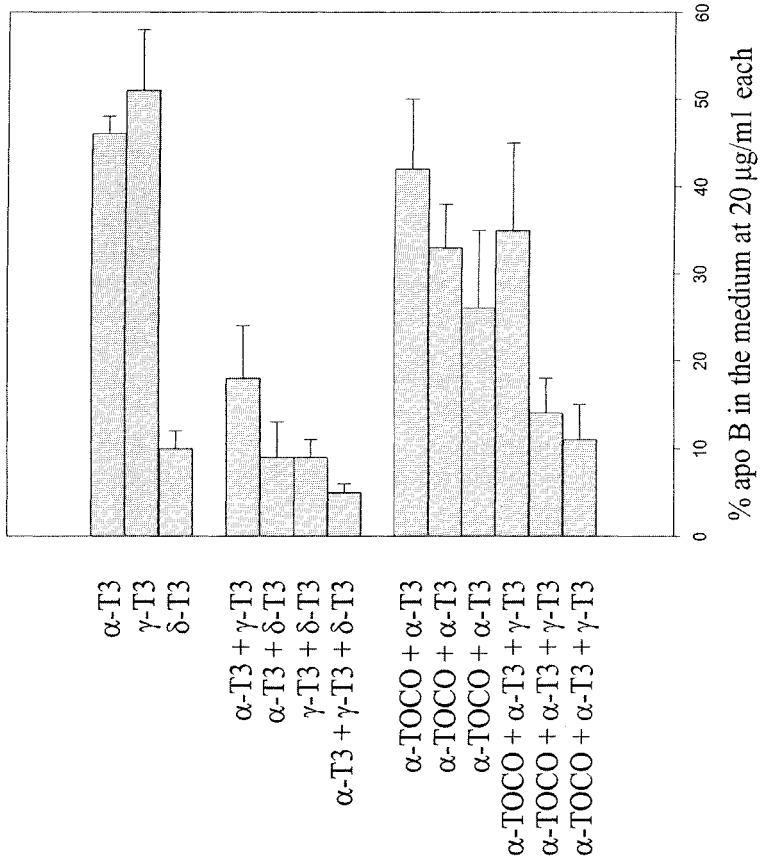


FIGURE 16.8 Changes in medium apo B concentration in response to tocotrienols and α-tocopherol.

postulating differences in their mechanisms of action. However, it is also possible that all tocotrienol isomers and α-tocopherol influenced each other's effects during the transport into hepatocytes, as suggested previously.[4] Further studies aimed to better understand the nature of tocotrienol/tocotrienol and tocotrienol/α-tocopherol interactions in cells should help to develop TRF-derived preparations with enhanced cholesterol-lowering potential.

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17 Dietary Fiber and Coronary Heart Disease

Thunder Jalili, Robert E.C. Wildman, and Denis M. Medeiros

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I. DIETARY FIBER CLASSIFICATION AND FOOD SOURCES

Most nutrition scientists are comfortable defining fiber as plant material that is generally resistant to human digestive enzymes. Another descriptor often used for fiber is "non-starch polysaccharides." However, one problem with this description is that the plant fiber lignins are actually polyphenolic in nature, not polysaccharide. Table 17.1 provides the percentage of the total weight of select foods that is attributable to fiber.

Fiber is typically subdivided into two groups based upon solubility in water. Soluble (water) fibers include pectin (pectic substances), gums, and mucilages, whereas the insoluble fibers include cellulose, hemicellulose, lignin, and modified cellulose. Table 17.2 presents these classes of fiber and lists some food sources for each type. Some of the better food sources of soluble fibers are fruit, legumes, oats, and some vegetables. Meanwhile, those foods noted to be richer sources of insoluble fibers include cereals, grains, legumes, and vegetables. The amount of fiber present within the human diet can vary geographically. In more industrially developed countries, such as the United States, fiber consumption is relatively lower than in other societies. For example, the average intake of fiber in the United States is only about 12 to 15 g daily. This consumption falls well below current recommendations of the World Health Organization of 25 to 40 g of fiber daily. The American diet tends to derive less than one half of its dietary carbohydrate intake from fruit, vegetables, and whole grains. On the other hand, the people of some African societies are known to eat as much as 50 g of fiber daily.

Digestible polysaccharides in plant foods such as starch, and to a much lesser degree glycogen in meats, have repeating monosaccharide units bonded by α1–4 linkages (Figure 17.1). These bonds are readily digested by amylase in both salivary and pancreatic secretions. Branch points in the starch and glycogen chains are joined through α1–6 linkages that are hydrolyzed by the enzyme α1–6 dextrinase (isomaltase) in pancreatic secretions. On the contrary, β1–4 linkage are formed by plants instead of α1–4 linkages between monosaccharides in fiberous polysaccharides (Figure 17.1). Both salivary and pancreatic amylase is unable to hydrolyze β1–4 covalent bonds efficiently.

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TABLE 17.1
Fiber Content of Select Foods

Food	Fiber (% weight)
Almonds	3
Apples	1
Lima beans	2
String beans	1
Broccoli	1
Carrots	1
Flour, whole wheat	2
Flour, white wheat	<1
Oat flakes	2
Pears	2
Pecans	2
Popcorn	2
Strawberries	1
Walnuts	2
Wheat germ	3

Source: Adapted from Wildman, R.E.C. and Medeiros, D.M., Carbohydrates, in *Advanced Human Nutrition*, CRC Press, Boca Raton FL, 2000.

TABLE 17.2
Fiber Types and Characteristics, Food Sources, and Bacterial Degradation

Types of Fiber	Characteristics	Food Sources	Degradation[a]
Soluble			
Pectins	Rich in galacturonic acid, rhamnose, arabinose, galactose; characteristic of middle laminae and primary wall	Whole grains, legumes, cabbage, root vegetables, apples	+
Gums	Composed mostly of hexose and pentose monomers	Oatmeal, dried beans, other legumes	+++
Mucilages	Synthesized by plant cells and can contain glycoproteins	Food additives	+++
Insoluble			
Cellulose	Structural basis for cell wall; only monomer is glucose	Whole grains, bran, cabbage family, peas, beans, apples, root vegetables	+
Hemicellulose	Component of primary and secondary cell walls; different types vary in monomer content	Bran, cereals, whole grains	+
Lignin	Composed of aromatic alcohols; cements, other cell wall components	Vegetables, wheat	0

[a] Denotes the degree of bacterial fermentation.

Source: Adapted from Wildman, R.E.C. and Medeiros, D.M., Carbohydrates, in *Advanced Human Nutrition*, CRC Press, Boca Raton FL, 2000.

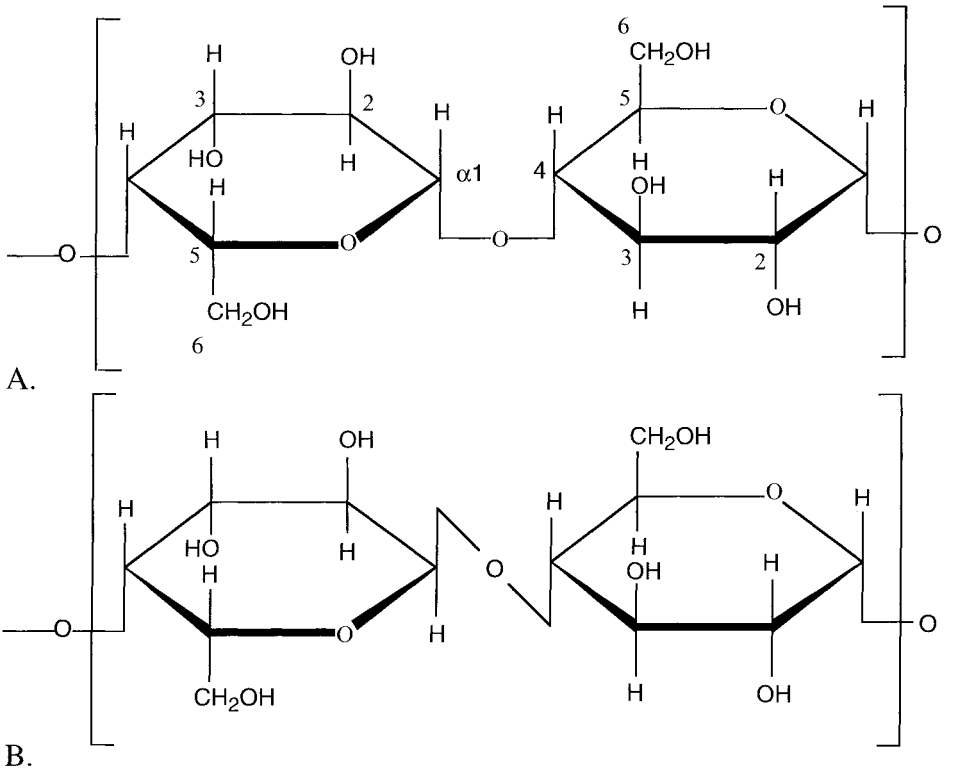

FIGURE 17.1 The α1–4 bond between glucose monomers of starch and glycogen (A) and the β1–4 linkage between glucose units in cellulose (B).

This renders such polysaccharides resistant to human digestive action. However, bacteria inhabiting the large intestine can indeed metabolize some polysaccharide fiber and create short-chain fatty acids (acetic, propionic, and butyric acids) as metabolites. These short-chain fatty acids, often referred to as volatile fatty acids (VFA), are a potential energy substrate for the mucosal cells of the large intestine. This is to say then that perhaps the notion that dietary fiber is not an energy source may need to be reconsidered. However, this point may only be significant in principle, as the energy contribution would be pretty small.

Cellulose is known to be the most abundant organic molecule on Earth. The molecular structure is similar to amylose in that it is made up of repeating units of the hexose glucose. However, again, the linkages will be β1–4 in nature. Cellulose is produced as a component of the plant cell wall by an enzyme complex called cellulose synthase. Once cellulose chains are formed, they quickly assemble with other cellulose molecules and form microfibrils that strengthen the cell wall. Cellulose, along with certain other fibers (hemicellulose and pectin) and proteins, is found within the matrix between the cell wall layers. This concept is somewhat similar to connective tissue matrix found within bone, tendons, and ligaments in humans. Hemicellulose is different from cellulose in that its monomers are heterogeneous. Hemicellulose will contain varied amounts of pentoses and hexoses covalently bound in a β1–4 linkage, as well as some branching side chains. Some of the more common and familiar monosaccharides in hemicelluloses are xylose, mannose, and galactose (Figure 17.2). Other monosaccharide subunits include arabinose and 4-*O*-methyl glucuronic acids.

Lignin stands alone as a fiber in that it is not a carbohydrate; yet it is considered an insoluble dietary fiber. Lignin is made up of aromatic polymers of chemicals from plant cell walls and provides plants with their "woody" characteristics. Lignin molecules are highly complex and

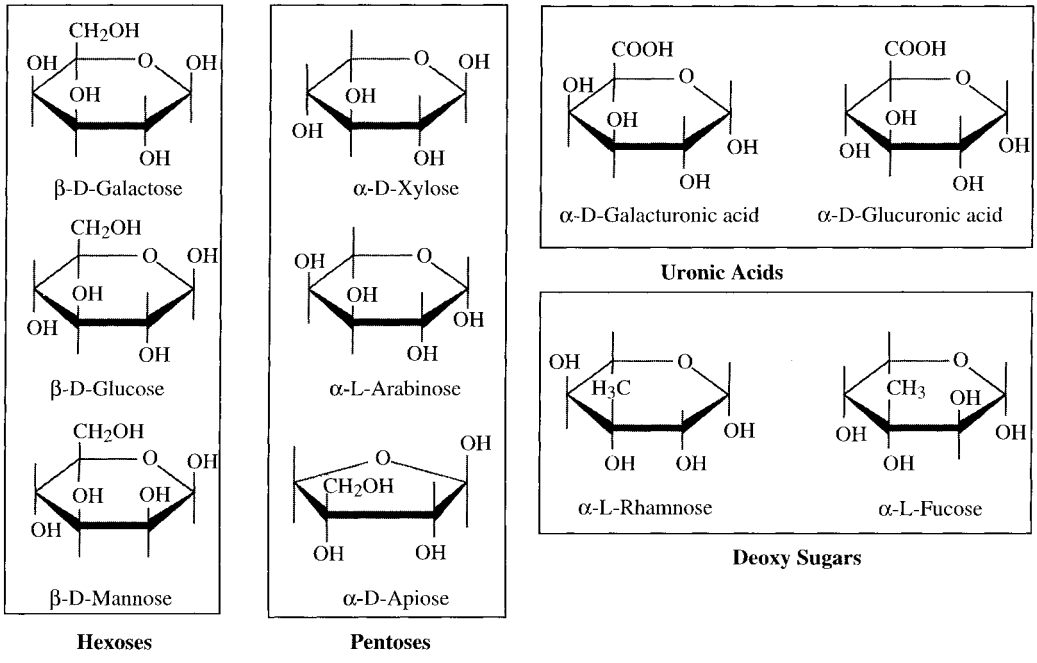

FIGURE 17.2 Carbohydrate monomers common to polysaccharide fibers.

variable polymers and are composed of three major aromatic alcohols: coumaryl, coniferyl, sinapyl. In plants lignin provide structure and integrity, thus allowing the plant to maintain its form. A typical lignin monomer is presented in Figure 17.3.

The soluble fiber pectin is composed mostly of galactouronic acid that has been methylated. These units are also connected by β1–4 linkages in pectin. The degree of methylation increases during the ripening of fruit and allows for much of the gel-formation properties of soluble fibers. Gums and mucilages are also soluble fibers and are composed of hexose and pentose monomers.

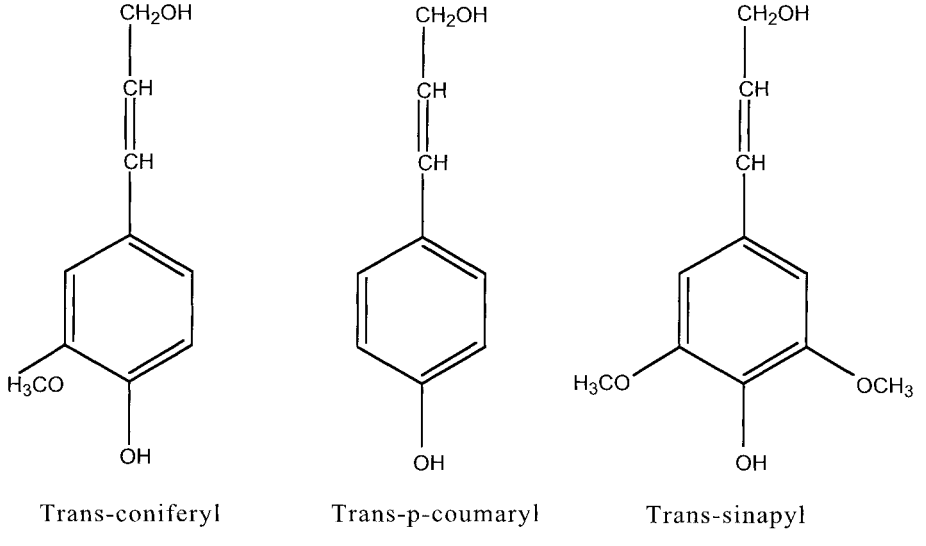

FIGURE 17.3 A typical phenolic monomers of a lignin molecule.

The physical structure and properties of these fibers are similar to pectin. Interestingly, gums are polysaccharides that are synthesized by plants at the site of trauma and appear to function in a manner similar to scar tissue in humans. Meanwhile, mucilages are produced by plant secretory cells to prevent excess loss of water through transpiration.

II. PHYSICAL AND PHYSIOLOGICAL PROPERTIES OF FIBER

The physiological attributes of fiber largely depend upon their physical characteristics, namely, the molecular design and solubility. Although the physiological influences of dietary fibers were once thought to be limited to the intestinal lumen, which is anatomically exterior, newer evidence suggests that derivatives of intestinal fiber metabolism can influence more internal operations as well. The physical characteristics of dietary fiber can produce various gastrointestinal responses depending upon the segment of the digestive tract. Among these responses are gastric distention, influences upon the rate of gastric emptying, and enhancement of residue quantity (feces bulk) and moisture content. Furthermore, dietary fiber can influence fermentation by bacteria in the colon as well as the turnover of specific bacteria species. More than likely, the bacterial population will increase due to fiber fermentation. Bacterial presence may contribute as much as 45% to the fecal dry weight. The influence of fiber upon fecal mass is presented in Table 17.3.

TABLE 17.3
Changes in Fecal Bulk Due to Different Dietary Fiber Sources

Food Item	% Increase in Fecal Weight
Bran	127
Cabbage	69
Carrots	59
Apple	40
Guar Gum	20

Source: Adapted from Wildman, R.E.C. and Medeiros, D. M., Carbohydrates, in *Advanced Human Nutrition*, CRC Press, Boca Raton FL, 2000.

Different fiber molecules are subject to varying levels of bacterial degradation in the colon (Table 17.4). For instance, pectin, mucilages, and gums seem to be almost completely fermented. Meanwhile, cellulose and hemicellulose are only partly degraded and the noncarbohydrate nature of lignin allows it to go virtually goes unfermented. The physical structure of the plant itself may be associated with the degree of degradation of food fibers by intestinal bacteria. As an example, fibers derived from fruits and vegetables appear to be, in general, more fermentable than those from cereal grains. VFAs, namely, acetic acid (2:0), proprionic acid (3:0), and butyric acid (4:0), are among the products of bacterial fermentation. As mentioned above, these fatty acids can be oxidized for ATP production in mucosal cells of the colon wall. Furthermore, these fatty acids are fairly water-soluble and can be absorbed into the portal circulation. Other products of bacterial fermentation of dietary fibers include hydrogen gas (H_2), carbon dioxide (CO_2), and methane (CH_4). These products may lead to occasional uncomfortable gas buildup in the colon that may occur with high fiber consumption. The presence of H_2 in the breath (hydrogen breath test) is often used clinically as an estimation of bacterial fermentation. Once produced, H_2 dissolves into the blood and circulates to the lungs.

Among some of their more interesting physical properties is the water-holding capacity or the *hydration* of fiber. The ability of the different fibers to associate with water molecules is largely

TABLE 17.4
Some Physical Properties of Different Fiber Types

Fiber Type	Action
Cellulose	Holds water, reduces colonic pressure, reduced transit time of digestion
Hemicellulose	Holds water, increases stool bulk, may bind bile acids, reduced colonic pressure, reduces transit time
Pectins, gums, mucilages	Slow gastric emptying, bind bile acids, increase colonic fermentation
Lignin	Holds water, may bind trace minerals and increase excretion, may increase fecal steroid levels

Source: Adapted from Wildman, R.E.C. and Medeiros, D.M., Carbohydrates, in *Advanced Human Nutrition*, CRC Press, Boca Raton FL, 2000.

attributable to the presence of sugar residues that have free polar groups (i.e., OH, COOH, SO, and C=O groups). These polar groups allow for the formation of hydrogen bonds with adjacent water molecules. It seems that pectic substances, mucilages, and hemicellulose have the greatest water-holding capacity. Cellulose and lignin also can hold water but not to the extent of other fibers. However, as soluble fibers are generally more fermentable, the associated water is liberated and absorbed in the colon. Thus, it is the insoluble fibers that hold onto water throughout the total length of the intestinal tract and give the fecal mass greater water content.

In the small intestine the hydration of fiber will allow for the formation of a gel matrix. Theoretically, the formation of a gel in the small intestine could increase viscosity of the food-derived contents and slow the rate of absorption of nutrients. It has been suggested this mechanism may slow of the rate of carbohydrate absorption and decrease the magnitude of the postprandial spike in blood glucose. This notion then may have application to individuals with diabetes mellitus, as discussed in this chapter.

III. RELATIONSHIP BETWEEN CHOLESTEROL LEVELS AND CHD

Coronary heart disease (CHD) is known to be the leading cause of death in the Western world according to the American Heart Association's 1996 Biostatistical Fact Sheet and reports from numerous medical organizations in Europe.[1] In contrast to popular dogma among the lay public, CHD is the leading cause of death among women as well.[2,3] Many risk factors can influence CHD, such as smoking, age, male sex, menopause, diabetes, serum cholesterol levels, and hypertension. Some of these risk factors are modifiable, such as smoking and serum cholesterol levels, and some are not, such as male sex or menopause. Among the most important risk factors that may be controlled are serum cholesterol levels. Many studies have established that high total cholesterol levels and low-density lipoprotein (LDL) cholesterol levels are risk factors for CHD and mortality.[4–6] The well-known Framingham Study was among the first to establish a statistical relationship between serum lipoproteins and CHD.[6] Other important studies using very large cohorts from the Multiple Risk Factor Intervention Trial (MRFIT), and from various countries, have since strengthened the notion that serum cholesterol is a risk factor for CHD.[4,5,7]

Elevated serum cholesterol levels can result from a variety of influences. Severely high serum cholesterol is usually due to familial hypercholesterolemia, a condition characterized by genetic defects in LDL receptor activity that result in accumulation of LDL cholesterol in the blood. Elevated serum cholesterol may also occur as a secondary effect of disorders such as diabetes, hypothyroidism, and in alcoholics. More commonly, cholesterol disorders are characterized by mild or moderate hypercholesterolemia and are generally dietary in origin. Dietary factors such as saturated fats, trans fatty acids, and fiber intake may all influence serum cholesterol levels.

A. Role of Fiber in Reducing Serum Cholesterol

Fiber has been implicated in reducing the risk for CHD. Many large epidemiological studies such as the Nurses' Health Study and the Scottish Heart Health Study have demonstrated a reduced risk for CHD in both men and women who consume higher amounts of dietary fiber.[8–10] The soluble fibers in particular are thought to exert a preventative role against heart disease as they appear to have the ability to lower serum cholesterol levels. A recent meta-analysis examining soluble fiber sources from pectin, oat bran, guar gum, and psyllium reported a small but significant reduction in serum cholesterol levels.[11] Many other studies have found that a high intake of soluble fiber results in decreased serum cholesterol levels.[12–19] These studies generally report a decrease in total cholesterol and LDL cholesterol with no changes in high-density lipoprotein (HDL) or triglycerides. Indeed, it is now recognized that soluble fiber is a viable intervention to reduce serum cholesterol levels in clinically significant amounts, thereby reducing a known risk factor for CHD.[20]

Oat bran in particular has received a great deal of attention as a fiber source with an appreciable level of soluble fiber that has been shown to reduce plasma cholesterol levels under controlled conditions.[14] Early studies examining the role of oats in reducing plasma cholesterol focused on supplementing oats without a great deal of dietary fat modification. In 1963, DeGroot and colleagues[21] published a study that supplemented rolled oats in the form of a bread to be consumed daily by 21 male volunteers between the age of 30 and 50. Over a 3-week period an 11% reduction in serum cholesterol was observed. Another early study by Anderson et al.[22] compared oat bran to fiber from beans in their ability to lower serum cholesterol. This study was conducted in a metabolic ward and did not use a low-fat diet. After consuming 17 g of soluble fiber per day for 3 weeks, a 19% decrease in total cholesterol and a 23% decrease in LDL cholesterol was observed. In more recent years scientists have been assessing the response of serum cholesterol to oat bran intake in conjunction with a low fat-diet. It has been found that a low-fat diet in conjunction with a high fiber (soluble) intake reduces cholesterol beyond the levels associated with a low-fat diet alone.[23]

A review of the literature demonstrates that oat bran has been repeatedly proved to play a role in reducing serum cholesterol levels and is generally recommended by the nutrition and medical community as an important part of the diet. A meta-analysis done by Ripsin and colleagues[24] reviewed results from ten trials and concluded that a significant amount of cholesterol reduction occurred when at least 3 g of soluble fiber from oat bran was consumed daily. Furthermore, it was observed that those subjects who had the most dramatic decrease in cholesterol levels were those who had the highest initial serum cholesterol concentrations to begin with.[24] In spite of the wealth of data supporting the role of oat bran in decreasing serum cholesterol, one issue remains ambiguous for the typical American consumer, that is the amount of oat bran needed to reduce serum cholesterol levels by clinically significant amounts. The lay public must realize that several servings of oat bran are required daily to reduce plasma cholesterol by an appreciable amount. Indeed, many of the studies that report significant decreases in serum cholesterol levels use very high intakes of soluble oat bran fiber. Most studies have used anywhere from 3.4 to 17 g of soluble fiber from oat bran to achieve total cholesterol and LDL-cholesterol reductions with the most severe declines observed with the highest use of soluble fiber. When one considers that the typical serving of instant oatmeal (0.5 cups) contains 1 to 2 g of soluble fiber, the reality of the dietary change involved becomes more apparent. In practice, it may be difficult for the average individual to consume levels of soluble fiber equivalent to the highest amounts used in certain studies. However, with moderate dietary changes it is possible to consume enough oat bran to fall in the lower range of experimental amounts previously used, which would result in a statistically significant reduction in serum cholesterol.

The long-term interest in oat bran has led to the identification of β-glucan as imparting a beneficial effect on cholesterol levels.[16] In addition to oat bran, yeast has also been identified as a concentrated source of β-glucan and is currently under investigation for its potential as a commercial additive in a variety of food products.[16] A relatively new product that is known by the patented

name Fibercel® is composed of purified β-glucan derived from the yeast *Saccharomyces cerevisiae*, the species found in baker's and brewer's yeast. This product is currently under investigation in clinical trials to establish its efficacy in treating individuals with high cholesterol levels. If proved successful, it could be used as an additive for foods such as salad dressing, frozen desserts, and cream cheese.

Recent studies using Konjac-mannan fiber (a highly soluble fiber also known as glucomannan) have also yielded very promising results by reducing risk factors for CHD. Subjects supplemented with a daily average of 23 g of Konjac-mannan in the form of biscuits experienced a lower total:HDL cholesterol ratio and LDL cholesterol, lower systolic blood pressure, and improved their glycemic control.[25] These results were significantly better than those achieved with an identical diet using wheat bran instead of Konjac-mannan diet, thereby demonstrating the effectiveness of the soluble fiber in influencing not only cholesterol, but other CHD risk factors, as well. Konjac-mannan fiber itself is well known for having among the highest viscosity of all the soluble fiber types. The use of Konjac-mannan fiber may also lead one to speculate that highly soluble fibers, such as Konjac-mannan, may be more effective at reducing cholesterol levels than other soluble fibers. It must be borne in mind, however, that the use of Konjac-mannan entails supplementation in existing foods, such as breads or biscuits, rather than eating an actual product such as oatmeal cereal. This may have practical relevance since it is far simpler for the typical consumer to buy instant cereal and eat it daily for breakfast than to buy Konjac-mannan fiber and supplement it in baked goods on a daily basis.

Other types of soluble fibers have been extensively studied for their ability to lower serum cholesterol amounts. Psyllium has received attention over the years as a soluble fiber that can reduce cholesterol levels. Psyllium is a plant whose stalks contain tiny seeds, also called psyllium, covered by husks, which is the source of the fiber. There is a great deal of soluble fiber in psyllium; in fact 71% of the weight of psyllium is derived from soluble fiber. In contrast, only 5% of oat bran by weight is made of soluble fiber; in other words, the soluble fiber in 1 tablespoon of psyllium is equal to 14 tablespoons of oat bran.

A number of animal studies have demonstrated that rats fed controlled diets supplemented with psyllium fiber experience a significant decrease in serum cholesterol levels.[26–28] A study done by Anderson et al.[26] even found reductions of up to 32% in cholesterol levels in rats fed 6% dietary psyllium. Many studies in humans have also found psyllium to be an effective agent.[12,13,19] Supplementation of 10.2 g of psyllium per day for 8 weeks in men consuming a 40% fat diet has resulted in a 14.8% reduction in total cholesterol and 20.2% reduction in LDL cholesterol.[19] Another study using higher amounts of dietary psyllium (15g/day) observed a change of LDL cholesterol from 184 mg/dl to 169 mg/dl.[29] A very recently published study has demonstrated that men with Type II diabetes supplemented with 10.2 g of psyllium daily for 8 weeks also experienced an 8.9% drop in total cholesterol and a 13% decline in LDL cholesterol.[30] This group of men with Type II diabetes displayed an improvement in glycemic control as well. Indeed, the results of a large meta-analysis done recently that examined 12 published and unpublished studies has concluded that consumption of dietary psyllium is linked with reductions in serum total and LDL cholesterol.[12] Even though psyllium has not achieved as much attention in the popular press compared with oat bran, there is evidence that it may actually be more effective as a dietary agent to lower cholesterol levels. Anderson et al.[26] compared ten different dietary fiber types in the rat model and found psyllium to be the most effective at lowering serum cholesterol levels. A study in humans using psyllium and oat bran demonstrated an equivalent reduction in total and LDL cholesterol when psyllium was used in one half the amount of oat bran. These studies could lead one to conclude that psyllium fiber may actually be more effective at reducing cholesterol levels and therefore could be consumed in lesser amounts to achieve desirable results. In fact, in 1998 the FDA approved labels on cereals supplemented with psyllium that state regular consumption of psyllium as part of a low-fat diet can reduce cholesterol levels.

B. Mechanisms for Lowering of Serum Cholesterol by Fiber

There are several possible mechanisms in which soluble fiber is thought to reduce serum cholesterol levels; many are related to the ability of soluble fibers to form viscous gels in the intestinal tract. Among these potential mechanisms are reduced cholesterol absorption in the presence of soluble fiber, increased excretion of bile acids, an alteration of bile acid type present in the gut, and possible influences of short-chain fatty acid production by intestinal flora upon cholesterol synthesis.

It has been proposed that soluble fiber reduces plasma cholesterol through its ability to bind bile acids in the gastrointestinal tract. As soluble fibers bind bile acids in the intestinal tract, micelle formation is altered and reabsorption of bile acids is subsequently impaired, resulting in the excretion of the fiber–bile complex through the feces. There are two classes of bile acids, primary and secondary. Primary bile acids (cholic and chenodeoxycholic acid) are those synthesized directly from the liver whereas secondary bile acids (deoxycholic and lithocholic acid) are produced after modification of primary bile acids by bacterial action in the colon. It has been demonstrated that consumption of oat bran increases the loss of bile acids by twofold and specifically increases the loss of deoxycholic acid (secondary bile acid) by 240% in human subjects.[31] It was also concluded that the pool of bile acids was not decreased, even though bile acid excretion is increased.[31] Another human study done with soluble fiber from psyllium found increased bile acid turnover of both primary bile acids as well.[29] These studies point to the fact that bile excretion is increased when high amounts of soluble fiber are eaten. Usually bile is reabsorbed in the large intestine and reused in emulsification of fats, however, since a constant pool is required the excreted bile must be replaced to keep bile levels adequate for digestive needs. Theoretically, this would indicate that bile acid synthesis would be increased under these conditions and indeed an increase in bile acid synthesis has also been observed in individuals consuming high amounts of soluble fiber.[29] Specifically, the synthesis of deoxycholic acid has been found to increase with consumption of a high-fiber diet. This may have further beneficial effects as deoxycholic acid has been shown to decrease absorption of dietary cholesterol.[32]

Replacement of bile can be achieved two ways: (1) more hepatic cholesterol can be dedicated for bile synthesis instead of being exported in the circulation as very low-density lipoprotein (VLDL), and (2) increased hepatic cholesterol demand will upregulate synthesis and activity of LDL receptors allowing for greater amounts of VLDL remnants and LDL to be removed from circulation. The overall effect of these alterations is a reduction in LDL and total cholesterol levels. With regard to the first point, data from animal studies demonstrate an increased rate of cholesterol synthesis in the livers of psyllium-fed hamsters.[28] Specifically, the enzymatic activity of HMG CoA reductase, the rate-limiting enzyme for hepatic cholesterol synthesis, is observed to be increased three- to fourfold in hamsters fed soluble fiber.[33] This effect is thought to be transcriptionally mediated as mRNA levels have been found to be similarly increased in the same model.[33]

Alterations of LDL receptor activity are also possible under the influence of psyllium fiber; however, this has been found to occur in experimental animals fed high-fat and high-cholesterol diets. Usually consumption of a high-fat diet tends to depress LDL receptor activity, but hamsters fed high-fat and high-cholesterol diets in conjunction with high dietary soluble fiber demonstrate a restoration of LDL receptor expression to normal levels.[33]

Examination of the effects of oat bran consumption reveals a divergence in the mechanism of action between soluble fiber from oats vs. that of psyllium. Both have the ability to bind to bile acids and facilitate their excretion; however, they differ in their secondary influence on hepatic cholesterol synthesis. As mentioned above, psyllium fiber fed to animals has been found to increase hepatic cholesterol synthesis. Paradoxically, soluble fiber from oat bran has been found to depress hepatic cholesterol synthesis.[34] Bacterial fermentation of soluble fiber from oats results in the production of short-chain fatty acids, specifically propionate, that are absorbed in the colon and travel to the liver via the portal vein. Data from *in vitro* studies demonstrate an inhibition of hepatic

cholesterol and fatty acid synthesis under the influence of propionate.[34] The apparent paradox of psyllium fiber increasing cholesterol synthesis and oat fiber decreasing cholesterol synthesis may be explained by the fact that psyllium is very poorly fermented by bacteria in the colon; hence, little propionate is produce to decrease hepatic cholesterol synthesis.

In the final analysis, it seems that oat bran may be able to reduce cholesterol levels in a two-part fashion: increasing bile loss and decreasing endogenous hepatic cholesterol synthesis, thus resulting in a shift of serum cholesterol for bile synthesis. Psyllium may reduce serum cholesterol levels through only one relevant mechanism, the loss of bile acids. Furthermore, in spite of the increase in HMG CoA reductase activity and cholesterol synthesis under the influence of psyllium, hepatic cholesterol content continues to be markedly reduced in animals fed a high-psyllium diet.[33] Therefore, it seems that this upregulation is barely enough to meet the demands of bile acid synthesis, and obviously not enough to contribute significantly to VLDL exportation and hence LDL cholesterol levels. As one can conclude after careful consideration of the cited studies in this section, even though the net effect of soluble fiber consumption is well established, the specific biochemical events that occur in cholesterol metabolism are still incompletely understood and require more thorough testing.

C. Other Relevant Considerations for Fiber and CHD Risk

Fiber has also been implicated in reducing risk for CHD through mechanisms other than plasma cholesterol modification. One such example is through modification of blood clotting ability. An enhanced clotting ability coupled with atherosclerosis increases the risk of developing an occlusion in the coronary arteries and subsequent myocardial infarction. The ability of the blood to clot is dependent upon fibrinogen levels and quality of the resulting fibrin network. Pectin has been found to influence the concentration and quality of fibrin networks in the blood and reduce the tensile strength of these networks. Pectin supplements have been shown to decrease the strength and quality of fibrin networks; these types of networks are thought to be less atherogenic than fibrin networks under normal conditions and thus may represent another vehicle for reducing risk for CHD.[35]

Whole grains have also been shown to be protective against CHD as demonstrated by an inverse relationship between whole grain consumption CHD.[36–38] However, it still remains unclear whether this association is due to the fiber content of whole grains or other components of whole grains such as phytochemicals, antioxidants, folate, vitamin B_6, monounsaturated fatty acids, or ω-3 polyunsaturated fatty acids that may act to reduce CHD risk. In spite of a certain degree of confusion regarding the individual contribution of whole grain–derived fiber in reducing CHD risk, the overall beneficial effect of whole grains in general should not be overlooked.

IV. HEALTH CLAIMS ASSOCIATED WITH FIBER AND CHD

The U.S. Food and Drug Administration (FDA) allows food manufacturers to use certain health claims related to the link between dietary fiber and a reduced risk of heart disease. For example, upon review of the research literature, the FDA recognizes the relationship between fruit, vegetables, and grain products that contain fiber, particularly soluble fiber, and a reduced risk of CHD. Foods that apply for related health claims would include fruit, vegetables, and whole grain breads and cereals. To qualify, foods must meet criteria for low saturated fat, low fat, and low cholesterol. The foods must contain, without fortification, at least 0.6 g of soluble fiber per reference amount, and the soluble fiber content must be listed on the label. The health claim must use the terms *fiber*, *dietary fiber*, *some types of dietary fiber*, *some dietary fibers*, or *some fibers* and *coronary heart disease* or *heart disease* in discussing the nutrient–disease link. The term *soluble fiber* may be added. A sample health claim may read:

Diets low in saturated fat and cholesterol and rich in fruits, vegetables, and grain products that contain some types of dietary fiber, particularly soluble fiber, may reduce the risk of heart disease, a disease associated with many factors.

More specific to soluble fiber, the FDA has to date reviewed and authorized two sources of soluble fiber (whole oats and psyllium) to be eligible for use of a health claim with regard to a reduction in the risk of CHD (Table 17.5). In doing so, the FDA acknowledges that in conjunction with a low-saturated-fat and low-cholesterol diet, certain soluble fiber foods may favorably influence blood lipid levels, such as total cholesterol, and thus lower the risk of heart disease. Some foods fitting the bill include oatmeal muffins, cookies, breads, and other foods made with rolled oats, oat bran, or whole oat flour; hot and cold breakfast cereals containing whole oats or psyllium seed husk; and dietary supplements containing psyllium seed husk. Again, in order for a food manufacturer to use such a health claim on a food label, the food must meet criteria for "low saturated fat," "low cholesterol," and "low fat." The food must provide whole oats in at least 0.75 g of soluble fiber per serving. Foods that contain psyllium seed husk must contain at least 1.7 g of soluble fiber per serving. In addition, a claim must indicate the daily dietary intake of the soluble fiber source necessary to reduce the risk of heart disease. Also the claim must indicate the contribution that one serving of the product will make toward that intake level. Further still, the soluble fiber content must be stated in the nutrition label. In the health claim the food manufacturer must state "soluble fiber" qualified by the name of the eligible source of soluble fiber and "heart disease" or "coronary heart disease" in describing the nutrient–disease association. A sample claim may read as follows:

Diets low in saturated fat and cholesterol that include 3 g of soluble fiber from whole oats per day may reduce the risk of heart disease. One serving of this whole-oats product provides 3 g of this soluble fiber.

Eating soluble fiber from foods such as psyllium as part of a diet low in saturated fat and cholesterol may reduce the risk of heart disease.

TABLE 17.5
Total and Soluble Fiber Content of Selected Cereal Brans

Crude Bran Source (100 g)	Total Dietary Fiber (g)	Soluble Fiber (g)
Wheat bran (1 2/3 cups)	42	3
Oat bran (2/3 cups)	16	7
Rice bran (1 cup)	22–24	3–9
Corn bran (1 1/4 cups)	85	2–3

Source: Adapted from Wildman, R.E.C. and Medeiros, D.M., Carbohydrates, in *Advanced Human Nutrition*, CRC Press, Boca Raton FL, 2000.

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18 Omega-3 Fish Oils and Lipoprotein Metabolism

Sidika E. Kasim-Karakas

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I. INTRODUCTION

Effects of n-3 fish oils on plasma lipids are well known. Omega-3 fish oil supplementation consistently decreases plasma triglyceride (TG), raises low-density lipoprotein (LDL) cholesterol, and has variable effects on high-density lipoprotein (HDL) cholesterol.[1–3] This chapter focuses on the newer information which has contributed to the understanding of the mechanisms of these lipid changes and newer approaches to the use of n-3 fish oils in treatment of hyperlipidemias. To put the effects of n-3 fish oils in perspective, a brief summary of the lipoprotein metabolism is provided.

II. LIPOPROTEIN METABOLISM

The major plasma lipids are TG, cholesterol, phospholipids, and free fatty acids (FFA). Lipids are insoluble in the plasma. Therefore, in the circulation FFAs are transported by albumin and TG, and cholesterol and phospholipids are carried in macromolecules called lipoproteins. Cores of the lipoproteins carry TG and cholesterol ester and the surfaces contain free cholesterol, phospholipids, and apoproteins (apo).

There are five major classes of plasma lipoproteins: chylomicrons, very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), LDL, and HDL. Chylomicrons and VLDL carry primarily TG, whereas LDL and HDL are the major cholesterol transporters. The IDL have approximately equal amounts of TG and cholesterol.

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Chylomicrons are synthesized in the intestine, and VLDL are synthesized in the liver. The structural apoprotein of chylomicrons is apo B 48, and that of VLDL is apo B 100. These TG-rich lipoproteins are hydrolyzed by lipoprotein lipase (LpL) and converted into smaller lipoproteins called remnants and IDL, respectively. The remnants and IDL are cleared from the circulation by the liver through mechanisms involving LDL-receptor-related protein (LRP), which binds to apo E and hepatic lipase (HL). Although all the chylomicron remnants are cleared by the hepatic uptake, some of the IDL are further catabolized to LDL. During this process the TG core is replaced by cholesterol ester and all of the apoproteins, except apo B-100, are removed.

Catabolism of LDL depends primarily on the receptor-mediated uptake. The LDL receptor binds the apo B 100. The LDL and the LDL receptor are internalized and catabolized in the lysosomes, releasing free cholesterol. Free cholesterol inhibits the key enzyme of cholesterol synthesis (3-hydroxy 3-methyl glutaryl-CoA reductase), creating a feedback mechanism to prevent excessive cholesterol production. Simultaneously, LDL-receptor synthesis is decreased and cholesterol esterification is stimulated. These feedback mechanisms tightly regulate the intracellular free cholesterol levels.

Production of HDL starts in the liver and intestine where the nascent HDL containing only a bilayer of phospholipid and apoproteins are formed. These immature particles are enriched with cholesterol ester through the action of lecithin cholesterol acyl transferase (LCAT), and by exchanging cholesterol for TG with other lipoproteins through the action of cholesterol ester transfer protein (CETP). These mature HDL deliver the cholesterol to the liver through the actions of HL and a specific receptor which recognizes apo AI, for excretion in the bile.

III. EFFECTS OF N-3 FISH OILS ON LIPOPROTEIN METABOLISM

A. Hepatic Production and Secretion of VLDL TG and VLDL apo B

Omega-3 fish oils decrease plasma concentration of VLDL TG, VLDL cholesterol, and VLDL apo B (Figure 18.1). Kinetic studies in humans, nonhuman primates, and small animal models

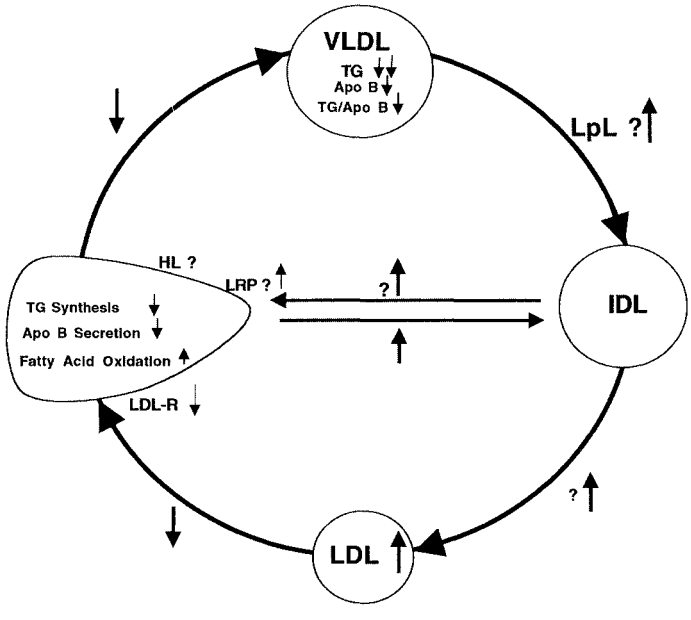

FIGURE 18.1 Omega-3 fish oil–induced changes in the metabolism of VLDL, IDL, and LDL. TG = triglyceride; LpL = lipoprotein lipase; HL = hepatic lipase; LDL-R = LDL receptor; LRP = LDL-receptor-related protein.

consistently demonstrate decreased VLDL TG and VLDL apo B secretion rates.[4,5] The hepatic secretion of TG is affected more than the secretion of apo B, thus resulting in the formation of VLDL particles containing relatively less TG.[5] Omega-3 fish oils can affect the rate of TG synthesis and secretion sequentially at three metabolic branching points: (1) the partitioning of cytosolic acyl coA between glycerol synthesis vs. oxidation, (2) the partitioning of diacylglycerol (DAG) between the formation of phospholipids vs. TG, and (3) the targeting of TG between the instant secretory pathway vs. the cytosolic storage pool. There is ample evidence to support that n-3 fish oils divert fatty acids into peroxisomal or mitochondrial oxidation[6–9] and induce carnitine palmitoyl transferase 1.[10,11] Activities of the key enzymes of DAG synthesis have been found both decreased and increased,[11,12] and it appears that n-3 fish oils divert the DAG metabolism from TG to phospholipid synthesis.[13,14] Furthermore, n-3 fish oils are preferentially incorporated into phospholipids rather than into TG.[14] *In vivo* studies show that n-3 fish oil feeding causes a 25% decrease in glycerol production from acyl CoA, 15% decrease in the incorporation of DAG into the TG pool, and a 20% decrease in hepatic TG secretion.[15]

Hepatic secretion of TG requires apo B. During fish oil feeding, although the VLDL TG and VLDL apo B secretion are decreased,[16–18] there is an increase in the liver weight and the liver TG pool. Since the preferential source of VLDL TG is the TG obtained from the lipolysis of cytosolic droplets, but not the newly synthesized TG, the increase in hepatic TG pool suggests that there may be a defect in the release of TG from the cytosolic pool.[19,20] The mechanisms underlying this finding are not known. It appears that this may be related to a decrease in apo B secretion, which occurs due to either a decrease in the synthesis or an increase in the intracellular destruction of apo B.[18,20] The recent research shows that microsomal TG transfer protein (MTP) prevents intracellular degradation of apo B by initiating its lipidation in the endoplasmic reticulum, and is responsible for the first step of VLDL synthesis.[21] To the author's knowledge it is not yet known whether n-3 fish oils regulate MTP.

B. Lipolysis of Triglyceride-Rich Lipoproteins

Since n-3 fish oil supplementation leads to the production of relatively TG-poor VLDL, these particles may lipolyze more efficiently. The available experimental evidence does not support this concept. In *in vitro* studies when n-fish-oil-enriched VLDL were hydrolyzed by using purified LpL and HL, both eicosapentanoic acid (EPA)- and docosahexanoic acid (DHA)-enriched particles had lower rates of hydrolysis as compared with soy oil. Release of fatty acids from EPA-enriched particles was even slower than those of the DHA.[22] In another study, when treated with LpL, n-3-enriched chylomicrons lipolyzed at a higher rate than the palm oil–rich particles, but at a comparable rate with olive oil–rich or corn oil–rich particles.[23] Although n-3 fish oils may not affect the lipolytic properties of the TG-rich lipoproteins, they can affect the activities of LpL and HL *in vivo*. In healthy subjects n-3 fish oils increased the activities of heparin releasable LpL and HL, whereas in patients with hyperlipidemia, only the LPL activity was increased.[24,25] When accelerated lipolysis is induced *in vivo* by intravenous infusion of heparin in patients with hyperlipidemia, there was no increase in the lipolysis of VLDL TG during n-3 fish oil treatment as compared to the olive oil placebo.[25] In Apo-E*3-leiden transgenic mice model, which has decreased clearance of chylomicron and VLDL remnants, n-3 fish oils increased VLDL clearance rate. When the rate of lipolysis was calculated by comparing the VLDL apo B vs. VLDL TG clearance rates, no change in the lipolytic rate was found.[26]

C. Metabolism of Chylomicron Remnants and IDL

The available evidence supports that n-3 fish oils increase the hepatic uptake of chylomicron remnants and IDL. In patients with diabetes, supplementation of EPA-ethyl ester decreased the concentration of remnantlike particles in the circulation.[27] In *in vivo* studies when n-3 fish oil–rich

chylomicrons were injected into normal rats, there was an increase in the biliary excretion of the chylomicron-derived cholesterol.[28] These findings were consistent with the results obtained from liver perfusion studies, where fatty acids from the n-3 fish oil–rich chylomicron remnants oxidized at a significantly higher rate. Labeled fatty acids in these remnants were incorporated primarily into the phospholipids. The recovery of the remnant cholesterol in the bile was also higher.[29] Furthermore, in the isolated hepatocytes, n-3 fish oil–rich remnants were internalized better than the olive oil– or palm oil–rich remnants. However, there was no significant change in the binding.[30] The mechanisms underlying the effects of n-3 fish oils on the remnant and IDL metabolism have not yet been identified.

D. Metabolism of LDL

In general, n-3 fish oils increase plasma LDL concentration. Both an increase in the production and a decrease in the clearance of LDL are responsible for the increase in LDL. The kinetic studies in hypertriglyceridemic patients with type 2 diabetes demonstrated that n-3 fish oils reduced plasma TG, VLDL TG, and VLDL apo B, but not total plasma apo B or IDL, and increased the LDL mass. The decrease in VLDL resulted from a decrease in the secretion of VLDL apo B, without a change in the residence time. There was also an oversecretion of apo B into the IDL fraction. The IDL particles became smaller. Although the transport of apo B in IDL remained unchanged, the fraction of IDL which was directly catabolized decreased, and the portion converted to LDL increased. The residence time of LDL did not change. Consequently, there was an increase in the LDL apo B mass.[31]

Studies in animal models and cell cultures consistently demonstrate that n-3 fish oils decrease the number of LDL receptors.[32] In addition, the changes in LDL structure and composition may also be important. In n-3 fish oil fed nonhuman primates, n-3 fish oil–rich LDL cleared less efficiently than the olive oil–rich particles. However, this was not a straightforward interaction, because in the olive oil–fed monkeys n-3 fish oil–rich LDL cleared as efficiently as the olive oil–rich LDL. It appeared that n-3 fish oils increased the cholesterol ester content of the LDL, which correlated strongly with the fractional catabolic rate.[33]

E. Metabolism of HDL

Omega-3 fatty acid supplementation has been associated with variable HDL cholesterol response. Plasma HDL concentration is regulated by several metabolic events, i.e., incorporation of cholesterol ester by LCAT, transfer of neutral lipids between lipoproteins by lipid transfer proteins and CETP, incorporation of surface lipids and apoproteins during the LpL-mediated lipolysis of TG-rich lipoproteins, removal of cholesterol ester and phospholipids by HL, and removal of cholesterol by a hepatic receptor recognizing apo AI. Decreased LCAT,[34,35] increased CETP,[36] decreased lipid transfer protein,[34,37] and increased or unchanged LpL and HL activities[24,25] all have been reported (Figure 18.2).

IV. EFFECTS OF N-3 FISH OILS ON GENE REGULATION IN ADIPOSE AND LIVER TISSUE

The effects of n-3 fish oils on the regulation of gene expression in the rat white adipose tissue has been investigated both in the subcutaneous and the retroperitoneal fat. The n-3 fish oils EPA and DHA were administered either individually, or in combination, and compared to the native fish oil. In the subcutaneous tissue, none of these dietary supplements changed either the cell size or the gene expression. However, in the retroperitoneal tissue, gene expression correlated directly with the cell size. The experimental groups who received the mixture of EPA and DHA, the native fish oil, and the DHA had smaller cell sizes. The animals who received EPA had cell

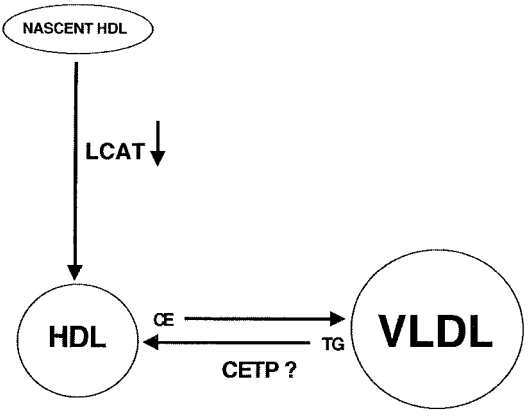

FIGURE 18.2 Omega-3 fish oil–induced changes in the metabolism of HDL. LCAT = lecithin:cholesterol acyltransferase; CETP = cholesterol ester transfer protein; CE = cholesterylester; TG = triglyceride.

sizes between the DHA-fed and lard/olive oil–fed groups. When compared to olive oil– and lard–fed group, n-3 fish oil–fed groups had lower mRNA levels for fatty acid synthase (FAS), hormone sensitive lipase, phosphoenolpyruvate carboxy kinase, LpL, and leptin.[38] In another study, both the control and genetically hypertriglycerdemic Wistar rats were fed a high-sucrose diet, either with or without n-3 fish oil. Sucrose feeding increased plasma TG concentration and the lipogenic enzymes both in the control and genetically hypertriglycerdemic animals. Omega-3 fish oil feeding suppressed the malic enzyme mRNA both in the liver and brown adipose tissue of the control rats. However, effects of n-3 fish oil were blunted in the genetically hypertriglycerdemic rats. Omega-3 fish oils also suppressed the mRNA for FAS in the liver and brown adipose tissue of the control rats, but failed to suppress it in the genetically hypertriglycerdemic rats. These findings suggested that n-3 fish oils may be more effective in controlling the dietary-induced hypertriglyceridemia.[39]

V. EFFECTS OF N-3 FISH OILS ON POSTPRANDIAL LIPEMIA

Although n-3 fish oils are potent inhibitors of the TG-rich lipoproteins of hepatic origin (VLDL), their effects on postprandial lipemia, or TG-rich lipoproteins of intestinal origin, have been variable. When effects of fish diet, n-3 fish oils, and DHA were compared with each other in healthy normal individuals, both the fish diet and n-3 fish oil supplementation decreased the fasting and postprandial lipemia. The DHA also had a significant lipid-lowering effect, although not as potent as the two other approaches.[40] In another study that compared the effects of EPA to DHA supplementation, there was a –19% decrease in postprandial triglyceride levels with EPA, and even a larger decrease (–49%) with DHA.[41] When the effects of n-3 fish oils on carbohydrate-induced hypertriglyceridemia were investigated in humans, concomitant fish oil intake prevented both the increase in TG and the decrease in HDL, but also exaggerated the postprandial response to a fatty meal.[42] These discrepancies may be due to the additional regulatory mechanisms which balance the fatty acid composition and production of chylomicrons.[43]

VI. SPECIFIC EFFECTS OF INDIVIDUAL N-3 FATTY ACIDS

The recent availability of purified ethyl esters of EPA and DHA provided the opportunity to identify their specific actions on the lipid metabolism. So far, the majority of the evidence supports that EPA, but not DHA, is the hypotriglyceridemic fatty acid. However, there is also evidence to the

contrary. When the effects of EPA, DHA, EPA/DHA combination, and olive oil placebo were compared in normal subjects, EPA decreased plasma TG, VLDL TG, and VLDL cholesterol and increased LDL cholesterol, whereas DHA did not affect these lipids. However, DHA increased the ratio of HDL_2 to HDL_3. Interestingly, while EPA supplementation did not cause any increase in DHA, there was a small retroconversion of DHA (22:6 n-3) to EPA (20:5 n-3).[44] At the mechanistic level, *in vivo* studies showed that EPA as well as DHA stimulated the rate-limiting enzyme in peroxisomal fatty acid oxidation: fatty acid CoA oxidase. Only EPA acutely increased hepatic fatty acid oxidation and mitochondrial 2,4-dienoyl-CoA reductase activity, along with the mRNA for these enzymes.[45] In the cultured rat hepatocytes, EPA stimulated the palmitic acid oxidation whereas DHA reduced it. Omega-3 fish oils also inhibited FAS. This effect was independent of the peroxisome proliferated activated receptor activity (PPAR).[46]

Effects of fish oils have also been compared with those of the flaxseed oil, which is rich in α-linolenic acid (18:3 n–3), the precursor fatty acid of EPA and DHA. Flaxseed oil supplementation caused small changes in plasma EPA and DHA concentrations but did not lower plasma TG, while fish oils reduced plasma TG significantly. Plasma LDL cholesterol and HDL cholesterol were not affected by either flax seed oil or n-3 fish oil treatment.[47]

VII. NEWER APPROACHES TO THE THERAPEUTIC USE OF N-3 FISH OILS

Recent research suggests that dietary proteins may modulate the effects of n-3 fish oils. In the rat, menhaden oil when administered along with soy protein had a better TG-lowering effect than when it was administered either with casein or cod protein. Casein and cod protein also increased the hepatic cholesterol concentration. However, the LpL activity in the epididymal fat pads was higher after cod protein than casein or soy protein.[48]

In several studies, n-3 fish oils have been combined with other known lipid-lowering nutrients. The primary purpose of this approach has been to counteract the n-3 fish oil–induced rise in the plasma LDL cholesterol. Two nutrients tested for this purpose are garlic and fiber. When the individual effects of garlic and n-3 fish oils were compared to that of placebo, n-3 fish oils reduced plasma TG but raised LDL cholesterol by 8%, whereas garlic reduced LDL cholesterol by 14%. In combination, garlic and n-3 fish oils reduced both plasma TG and LDL cholesterol.[49] Another study that investigated the effects of n-3 fish oil and garlic combination reported a 11% decrease in cholesterol and a 34% decrease in TG concentrations when compared with the placebo.[50] In noninsulin-dependent patients with diabetes effects of combination of fiber with n-3 fish oils have been investigated. It was noted that n-3 fish oil alone lowered TG, however, did not have any beneficial effects on LDL or HDL. Addition of fiber lowered LDL cholesterol and potentiated the TG-lowering effect of fish oils. There was also a decline in the plasma fatty acid concentration.[51]

Omega-3 fish oils have also been used to potentiate the effects of lipid-lowering medications. As a TG-lowering treatment, when efficacy of n-3 fish oils was compared with gemfibrozil directly, gemfibrozil was found to have a larger effect (–39% vs. –18%). Similarly, gemfibrozil reduced cholesterol by –13% vs. n-3 fish oils by –6%, apo B by –17% vs. n-3 fish oils by –10%, and LDL cholesterol by –15%, whereas fish oil had no effect.[52] When compared with fenofibrate, n-3 fish oils achieved a similar TG-lowering effect in patients with familial hypertriglyceridemia and dysbetalipoproteinemia.[53] Omega-3 fish oils have also been administered with simvastatin to patients with combined hyperlipidemia to achieve further TG lowering.[54]

In summary, recent research has improved our understanding of the effects of n-3 fish oils at a more mechanistic level. Availability of the ethyl esters of individual n-3 fatty acids, EPA and DHA, for human use should encourage further research to identify their specific metabolic actions and therapeutic potentials.

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19 Lipid Oxidation in Biological Systems Enriched with Long Chain n-3 Fatty Acids

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I. INTRODUCTION

The consumption of fish has been shown in numerous studies,[1–11] but not all,[12–15] to be associated with decreased mortality from coronary heart disease. This cardioprotective effect is thought to be derived primarily from the long-chain polyunsaturated fatty acids, eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), found in fatty fish. Paradoxically, as these fatty acids are oxidized readily in chemical systems and foods, there is concern that their consumption could be accompanied by increased *in vivo* lipid peroxidation, which may, in turn, contribute to carcinogenesis, atherosclerosis, inflammation, and numerous other diseases and disorders.[16] However, studies to examine the level of lipid peroxidation in biological systems enriched with EPA and DHA have produced contradictory findings.

The mechanism used to explain the oxidation of EPA and DHA in heterogeneous multiphase biological systems, such as those found in lipoproteins and membranes, is based on the autoxidation mechanism of unsaturated fatty acids in homogeneous chemical systems. Resolving the contradictory observations about oxidation of EPA and DHA *in vivo* requires an understanding of classical autoxidation kinetics. The purpose of this chapter is to review lipid oxidation in homogeneous and

0-8493-8734-5/01/$0.00+$.50
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biological systems. Since the method used to measure the extent of lipid oxidation markedly influences the conclusions drawn, several methods will be briefly discussed. As vitamin E is the major lipid-soluble autoxidant, its role in oxidation of EPA/DHA-enriched systems will be addressed. Finally, a mechanism for the oxidation of EPA and DHA in biological systems in which their role is more protective than destructive is proposed.

II. OXIDATION OF UNSATURATED FATTY ACIDS IN HOMOGENEOUS SYSTEMS

More than 50 years ago, Holman and Elmer[17] demonstrated that in homogeneous systems containing only one fatty acid or its methyl ester, the rate of oxidation of biologically relevant fatty acids of the n-6 and n-3 series increased if there were a double bond adjacent to the hydrogen atom being removed. A hydrogen atom adjacent to a double bond is an allylic hydrogen; it is a *bis*-allylic hydrogen if there are double bonds on each side of the hydrogen. Holman and Elmer[17,18] measured autoxidation of oleate (18:1n-9), linoleate (18:2n-6), α-linolenate 18:3(n-3), arachidonate (20:4n-6), and DHA in air at 37°C by following the rate of oxygen uptake as the samples spontaneously oxidized. Linoleic acid was 40 times more reactive than oleic acid and the relative maximum rates of oxidation for the other fatty acids increased as the number of *bis*-allylic hydrogen atoms increased.

Cosgrove and colleagues[19] measured the rate of oxidation of methyl esters of various polyunsaturated fatty acids (PUFA) dissolved in chlorobenezene. They used thermally labile azo initiators, 2,2′-azobis(2-methylpropionitrile) (AIBN) or 2,2′-azobis(2,3-dimethylvaleronitrile) (DMVN), which decompose to give radicals at a known and constant rate. Measuring oxygen absorption, they confirmed that the oxidizabilities of the PUFA increased as the number of *bis*-allylic hydrogen atoms increased. The rate approximately doubled for each *bis*-allylic hydrogen atom present in a fatty acid.

Recently, Yeo and colleagues[20] compared the free radical–mediated oxidation of 13 fatty acids, each having a different number of carbon atoms and *bis*-allylic hydrogen atoms. They measured malondialdehyde (MDA) by gas chromatography/mass spectrometry (GC/MS).[21] The amount of MDA produced depended on the total number of *bis*-allylic hydrogen atoms, as well the total lipid chain length. When oxidized with superoxide radical, fatty acids that contained 22 carbons oxidized more readily than those that contained 20, which, in turn, oxidized more readily than those that contained 18. Within the 22-carbon series, the order of oxidative susceptibility was 22:6n-3 > 22:4n-6 > 22:3n-3. However, in the series that contained 20 carbons, the order was 20:3n-6 > 20:4n-6 > 20:5n-3 > 20:3n-9. EPA was less susceptible to oxidize than anticipated based on its *bis*-allylic hydrogen atoms.

III. MECHANISM OF OXIDATION OF UNSATURATED FATTY ACIDS IN HOMOGENEOUS SYSTEMS

Based on the work discussed above and other similar studies,[22–24] the now well-known mechanism for the autoxidation of purified fatty acids in a homogenous system was derived. Lipid peroxidation in a chemical system is a free radical process consisting of three stages: initiation, propagation, and termination.[25] Halliwell and Gutteridge[16] define a free radical as "any species capable of independent existence that contains one or more unpaired electrons." In a completely peroxide-free system, initiation of lipid peroxidation occurs by the abstraction of a hydrogen atom from a methylene group ($-CH_2-$) on a fatty acid (RH in Figure 19.1, Reaction 1) by radical species ($X^\bullet$ in Figure 19.1, Reaction 1) such as the hydroxyl radical ($OH^\bullet$) or those generated by AIBN or 2,2′-azobis(2,3-dimethylvaleronitrile) (AMVN). The resulting molecule is a carbon-centered free radical ($R^\bullet$ in Figure 19.1, Reaction 1). This radical, in turn, reacts very rapidly with molecular oxygen to form a peroxyl radical, also called peroxy radical ($ROO^\bullet$) (Figure 19.1, Reaction 2). The peroxyl

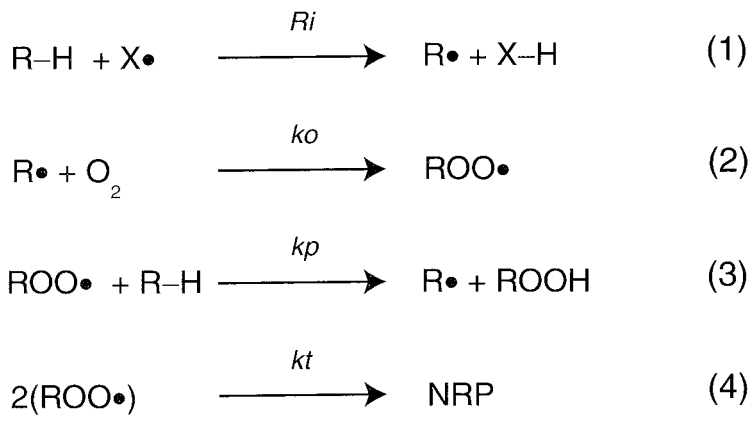

FIGURE 19.1 Theoretical model of the oxidation of a fatty acid by a free-radical mechanism. RH represents a fatty acid; X• any free radical capable of abstracting a hydrogen from RH; R• a free radical of the fatty acid; ROO• the peroxyl radical; ROOH a lipid hydroperoxide. R_i is the rate constant for the initiating event; k_o, k_p, and k_t are the rate constants for their respective reactions.

radical abstracts a hydrogen atom from another unsaturated fatty acid to make a new carbon-centered radical and a lipid hydroperoxide (ROOH), also called lipid peroxide (Figure 19.1, Reaction 3). These latter two terms are often used interchangeably. However, lipid peroxide includes the cyclic products that can be made as well as the hydroperoxides.[16] Because a new free radical is formed, a chain reaction is created, resulting in many lipid peroxides from one initiating event. Reaction 3, which is slower than Reaction 2, is the rate-determining step. The chain reaction is terminated when the chain-propagating species react with other free radicals or antioxidants to form nonradical products (NRP) (Figure 19.1, Reaction 4). Among the most important of the antioxidants is α-tocopherol. It rapidly donates a hydrogen atom to the peroxyl radical forming a lipid peroxide and becoming a radical itself in the process (Figure 19.1, Reaction 3). The tocopherol radical, since it is stabilized by resonance of its electrons, is less reactive than the original peroxyl radical. From these reactions, assuming that oxygen is present in sufficient quantities and steady-state conditions prevail, i.e., the concentrations of R• and ROO• do not change, the rate of oxygen consumption is given by the following expression:

$$-d[O_2]/dt = \{k_p/(2k_t)\}^{1/2}[RH]R_i^{1/2}$$

where k_p and k_t are the rate constants for the propagation and chain terminations steps, respectively, and R_i is the rate of chain initiation. The ratio of $k_p/(2k_t)^{1/2}$ represents a quantitative expression of the ease with which a fatty acid can be oxidized and is commonly called its oxidizability.[19]

Several different lipid hydroperoxides can be formed by the oxidation of each fatty acid. For example, the oxidation of oleic acid yields four products because a hydrogen atom can be abstracted from either of the two allylic carbons, carbon-8 or carbon-11, resulting in two delocalized three-carbon allylic radicals (Figure 19.2). As both ends of each of these intermediates have free radical characteristics, oxygen has four potential sites at which to react — carbons 8, 9, 10, or 11 — resulting in a mixture of four allylic hydroperoxides. In addition, geometric isomers are formed because the radical can assume different stereochemical conformations under different reaction conditions, further complicating the array of products.[26] [*Note:* Lipid peroxides are usually named with International Union of Pure and Applied Chemistry (IUPAC) nomenclature rather than the system favored by lipid chemists. In the IUPAC system the first carbon counted in locating the position of the first double bond is the carboxylate carbon. Lipidologists count the methyl terminus as the first carbon. The IUPAC convention is used in Figures 19.2 through 19.4.]

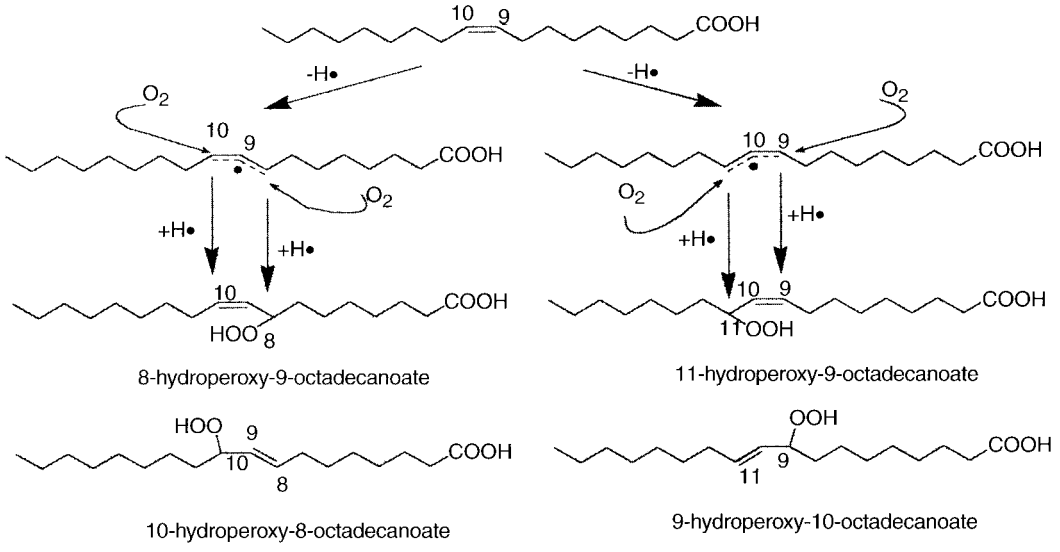

FIGURE 19.2 Mechanism for the formation of lipid hydroperoxides from oleic acid, 18:1n-9 (9-octadecanoate), resulting in four products. A hydrogen atom can be abstracted from either of the two allylic carbons, carbon-8 or carbon-11. Two delocalized three-carbon allylic radicals form. Oxygen can react at carbons 8, 9, 10, or 11, forming a mixture of four allylic hydroperoxides.

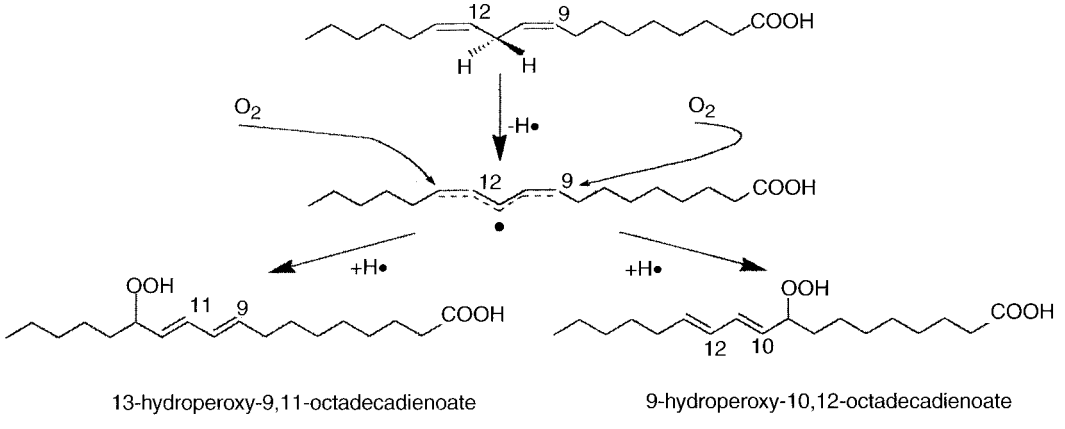

FIGURE 19.3 Mechanism for the formation of lipid hydroperoxides from linoleic acid, 18:2n-6 (9,12-octadecadienoate). A pentadienyl radical is formed when a hydrogen atom is abstracted from carbon-11. Oxygen can react with either carbon-9 or carbon-13, forming a mixture of two lipid hydroperoxides.

When linoleic acid is oxidized (Figure 19.3), the most reactive hydrogen is on the *bis*-allylic methylene group at carbon-11. A pentadienyl radical, which is stabilized by resonance, is formed. Because oxygen can react with either carbon-9 or carbon-13 on this radical, a mixture of two lipid hydroperoxides is formed. The resulting product, a conjugated diene, contains two adjacent double bonds. The final molecule is a dienoic hydroperoxide.

Oxidation becomes more complex as the number of *bis*-allylic hydrogen atoms increases. The peroxidation of α-linolenic acid gives four trienoic hydroperoxides, as well as cyclic peroxides such as hydroperoxy epidioxides and hydroperoxy bicycloendoperoxides (Figure 19.4). Peroxidation of arachidonic acid yields six conjugated diene hydroperoxides, EPA yields eight, and DHA yields ten. In addition, these three fatty acids form hydroperoxy bicycloendoperoxides.

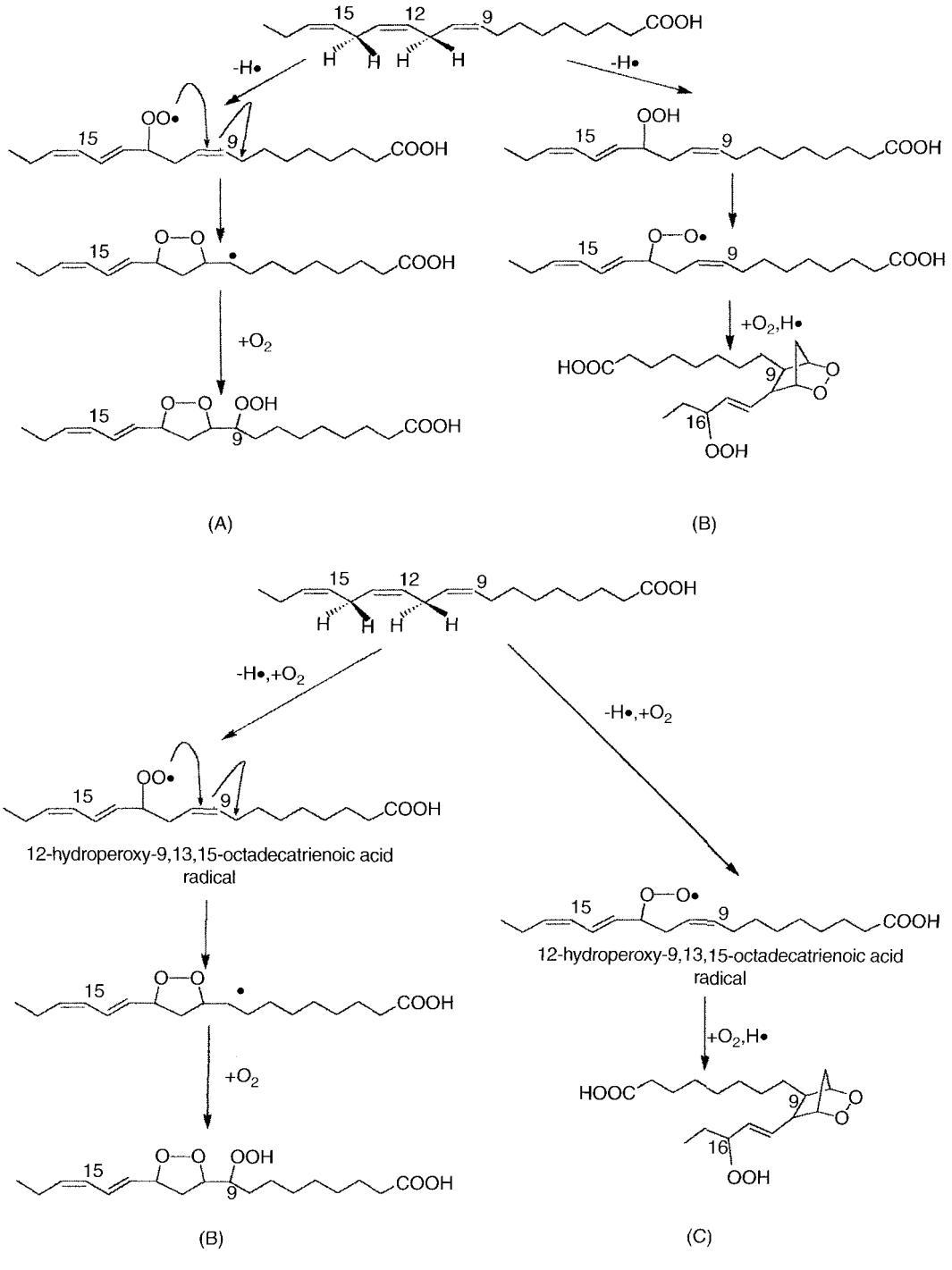

FIGURE 19.4 Mechanism for the formation of lipid peroxides from α-linolenic acid, 18:3n-3. (A) Formation of four trienoic hydroperoxides. (B) Formation of hydroperoxy epidioxide from 12-hydroperoxide of α-linolenic acid by internal 1,3-cyclization and subsequent oxidation. (C) Formation of hydroperoxy bicyclo endoperoxide from 12-hydroperoxide of α-linolenic acid by internal 1,5-cyclization and subsequent oxidation and hydrogen atom abstraction. Similar reactions to (B) and (C) would occur with the 13-hydroperoxide from α-linolenic acid.

Cyclic peroxides can be formed from PUFA by abstracting a hydrogen atom from another *bis*-allylic carbon on the same molecule. For example, the 12- and 13-hydroperoxides formed from α-linolenic acid rapidly undergo 1,3-cyclization and further oxidation to form five-membered hydroperoxides.[26] They can also undergo 1,5-cyclization and further oxidation to form 9- and 16-hydroperoxy bicycloendoperoxides, six-membered rings. Similarly, cyclization of lipid hydroperoxides can form from arachidonic acid, EPA, and DHA.

Although lipid peroxides are usually stable at room temperature, they will decompose to form a complex array of products on heating or in the presence of transition metal ions at physiologically relevant temperatures. For example, alkoxyl radicals (RO•), also called alkoxy radicals, form by decomposition of hydroperoxides.[16] Alkoxyl radicals can, in turn, react with an unsaturated fatty acid to make an alcohol and another carbon-centered free radical. Alternatively, the alkoxyl radical can react with a lipid hydroperoxide to generate another peroxyl radical. In either event, a radical is made that can further propagate the chain reaction.

IV. MEASUREMENT OF EXTENT OF LIPID OXIDATION

The extent of oxidation can be monitored by following the loss of oxygen as was done by Holman and Elmer,[17] Cosgrove and colleagues,[19]or more recently by Bonanome and colleagues[27] using an oxygen electrode, the loss of individual fatty acids by GC,[28,29] or the formation of various products. The products measured may occur in the initial stages of oxidation, as do free radicals, conjugated dienes, and lipid peroxides, or later, as do the degradation products of the lipid peroxides.

The presence of carbon-centered free radicals can be detected by electron paramagnetic resonance (EPR) spectrometry using a spin trap such as α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone (POBN). Although this is a very specific assay, because of the high cost of the equipment used and the expertise required to run the equipment, it has not been used frequently to measure lipid oxidation.The conjugated dienes that are made when a hydrogen atom is abstracted from a PUFA absorb ultraviolet light around 230 to 235 nm. Often the *in vitro* or *ex vivo* oxidation of low-density lipoprotein (LDL) is monitored by measuring the continuous production of conjugated dienes in the presence of a catalyst such as copper.[30] With this technique three variables can be obtained. The first is the lag phase during which time there is almost no production of conjugated dienes and the LDL-associated antioxidants are consumed. The second phase is the rate of production of conjugated dienes, usually referred to as the rate of oxidation of LDL, and is characterized by a rapid increase in diene formation as the fatty acids oxidize. The third phase is the maximum production of conjugated dienes. After the third phase, the absorbance measured quickly decreases as the conjugated dienes decompose. Although the lag time is frequently the only one of these three variables reported, the other two may also provide critical information.

Perhaps one of the most specific and sensitive assays to measure lipid hydroperoxides is by postcolumn chemiluminescent detection using high-pressure liquid chromatography (HPLC).[31] In this method, lipid hydroperoxides in the presence of microperoxidase presumably form alkoxyl radicals, which in turn react with isoluminol to emit light that is detected by a chemiluminescence detector. Cyclic peroxides are not detected.[32] Since antioxidants present in biological samples interfere with the production of chemiluminescence by scavenging free radical intermediates, the lipid hydroperoxides are separated from the antioxidants by HPLC. This assay has been particularly useful in detecting lipid hydroperoxides in lipoproteins.

Another method to measure lipid hydroperoxides, although less specific and sensitive than the postcolumn detection by HPLC method, uses a spectrophotometric procedure. In this method, hemoglobin catalyzes the reaction of lipid hydroperoxides with a methylene blue derivative, forming methylene blue, which can be measured colorimetrically. This method has been used chiefly in plasma, although it can be adapted for use in tissue samples.[33]

Recently, the cyclic compounds formed from the oxidation of PUFA, especially arachidonic acid, have been used to estimate the extent of lipid peroxidation.[34] The compounds formed from arachidonic acid by this nonenzymatic oxidation are very similar structurally to the prostaglandin $F_{2\alpha}$, which is formed when arachidonic acid is oxidized by cycloxygenase. Because of this similarity in structure, the compounds formed from arachidonic acid are known as F_2-isoprostanes and are currently considered to be particularly good markers for *in vivo* lipid peroxidation. However, they do not measure oxidation of EPA or DHA. Nourooz-Zadeh and colleagues[35,36] showed that other similar compounds, which they named F_3-isoprostanes, are formed during peroxidation of EPA. Roberts and colleagues[37] found that the oxidation of DHA *in vitro* yielded F_2-like compounds, which they termed F_4-neuroprostanes. Interestingly, they found increased levels of these neuroprostanes in cerebrospinal fluid from patients with Alzheimer's disease compared with normal controls, suggesting that lipid peroxidation may contribute to this neurodegenerative disorder. The use of the F_3- and F_4-isoprostanes to measure lipid peroxidation after dietary interventions with these fatty acids remains to be explored.

Many aldehydes, ketones, and hydrocarbons are made from the decomposition of the lipid peroxides.[16] These products are often used to measure the extent of lipid peroxidation, even though they may be far removed from the initial lipid peroxides and often reflect only a small fraction of the total decomposition products. Because of this, the conclusions that can be drawn from these measures of lipid peroxidation are often ambiguous. The aldehyde used most frequently to quantitate the amount of lipid peroxidation that has occurred is MDA. It is formed primarily by the oxidation of fatty acids with three or more double bonds, such as α-linolenic acid, arachidonic acid, EPA, and DHA, and to only a limited extent from oxidation of linoleic acid. Pryor and colleagues[38] proposed that MDA was obtained from the degradation of hydroperoxy epidioxides or hydroperoxy bicycloendoperoxides.

The usual procedure to measure MDA is to heat it extensively in the presence of thiobarbituric acid (TBA). An adduct is formed that has a molar ratio of 1 MDA to 2 TBA and can be measured colorimetrically or fluorometrically. However, this assay is fraught with many well-recognized problems.[39] It lacks specificity because TBA, in addition to reacting with MDA, will react with numerous other compounds, including some carbohydrates, amino acids, and nucleic acids, to generate adducts that absorb at a wavelength similar to where the MDA:TBA_2 adduct absorbs. Because of this lack of specificity, this assay is referred to as the thiobarbituric reactive substances assay (TBARS). Further, the MDA that is measured by this assay can come from various sources. *In vivo* not only is MDA formed by a free radical process but also enzymatically by cycloxygenase. It can also be formed *in vitro*. Heating the sample to form the adduct will cause additional lipid peroxides to form, which can then decompose to form MDA. Consequently, the value measured by the TBARS assay will suggest that more lipid peroxidation has occurred than, in fact, actually has. The specificity of this assay is improved if the MDA:TBA_2 adduct is separated from interfering compounds by HPLC[40] before being measured and if an antioxidant such as di-*tert*-butyl-4-methylphenol (butylated hydroxy toluene, BHT) is used to retard formation of MDA during the analytical steps. A further improvement in measuring the concentration of MDA was recently devised by Liu and colleagues.[21] They derivatized MDA with pentafluorophenyl hydrazine at room temperature and measured the amount present by GC/MS, thus decreasing oxidation during the sample preparation and increasing specificity of the assay.

In addition to MDA, other aldehydic end products, including formaldehyde, acetaldehyde, propanal, butanal, pentanal, hexanal, octanal, and nonanal, are formed by lipid oxidation. Several of these aldehydes have also been used to evaluate the extent of lipid peroxidation.

The hydrocarbons pentane and ethane can be made from n-6 and n-3 fatty acids, respectively, and their concentration in expired air can be used to monitor the oxidation of these two series of fatty acids.[41] However, the production of these two compounds is very low in comparison with other degradation products such as MDA and conjugated dienes. Both gases are present normally

in breath so the small amounts resulting from lipid peroxidation are difficult to separate from the background material. The gases are measured by GC, which makes the assay labor-intensive and expensive. Despite these drawbacks, measuring their concentration in expired air may be an appropriate, noninvasive way to discriminate between oxidation of n-3 and n-6 fatty acids.

No method of measuring the extent of lipid peroxidation can be considered a "gold" standard. It is particularly difficult to determine the extent of oxidation of individual fatty acids. Prevailing opinion is that it is necessary to measure at least two markers of lipid peroxidation simultaneously to gain an understanding of the chemical processes occurring. It is also imperative that the chemical entity measured be explicitly stated rather than referring to all measurements with one collective term, *lipid peroxidation.*

V. OXIDATION OF EPA/DHA-ENRICHED BIOLOGICAL SYSTEMS

Although the current understanding of the oxidation of unsaturated fatty acids in biological systems is based on classical autoxidation kinetics, this mechanism may not accurately account for the complexity that exists *in vivo*. The striking difference between oxidation of unsaturated fatty acids in homogeneous purified systems and in biological systems is that, in the latter, the fatty acids are in discrete microenvironments. For example, in lipoproteins they are esterified to phospholipids that form the outer boundary or esterified to cholesterol or glycerol in the core. The outer boundary makes a polar phase and the core makes a nonpolar phase. In membranes, fatty acids are present as the acyl groups of phospholipids in the bilayer that surrounds the cell or its organelles. The phospholipid acyl chains in the interior of the bilayer form a more nonpolar phase than the phospholipid surface head groups. Despite the fact that in homogeneous chemical systems it appears fairly conclusive that the oxidative susceptibility of fatty acids is highly dependent on the number of *bis*-allylic hydrogen atoms, that is not the case in more complex biological systems. Numerous studies have shown that after enriching a biological system with EPA or DHA, lipid oxidation may increase, not change or decrease.

A. Increased Oxidation in EPA/DHA-Enriched Biological Systems

1. Influence of Fatty Acid Composition on Cellular Lipid Peroxidation

Experiments conducted in the early 1990s by Buettner, Burns, Spector, and colleagues addressed the question of relative oxidizability of cells enriched with EPA and DHA.[42–46] Cellular fatty acid content was modified in culture by incubating cells (L1210 murine leukemia cells,[46] U937 human monocytic leukemia cells,[45] and endothelial cells,[42] both porcine pulmonary artery endothelial cells and bovine aortic endothelial cells) with oleic acid, α-linolenic acid, γ-linolenic acid (18:3n-6), arachidonic acid, EPA, adrenic acid (22:4n-6), and DHA. They measured radical formation by EPA using POBN as a spin trap. They concluded that the spin adduct formed between the trap and the radicals contained short-chain alkyl radicals (R•), secondary products derived from the cleavage of the alkoxyl radical formed from peroxidized fatty acyl chains.[45] They found that the amount of lipid radicals generated increased with the total number of *bis*-allylic positions in the cellular lipids. Surprisingly, the oxidation products formed by the intact cells were not detected in the intact cell pellet but only in the extracellular medium.[43] The authors reasoned that most of the radicals were formed in the microenvironment of the cell surface where they had ready access to the medium. These results indicated that not only did increased oxidation occur in heterogeneous biological systems enriched in EPA and DHA but that the increase was possibly greater than that which occurred in homogeneous chemical systems.

The concept of lipids forming microenvironments and this influencing their oxidation was not new.[47] Phospholipids, in particular dilinoleoylphosphatidylcholine (DLPC), aggregate in nonpolar solvents such as benzene and chlorobenzene forming reverse micelles.[48] Reverse micelles are

particles in which the polar head groups of the phospholipids are located in the center of the particle while the nonpolar chains are oriented outward, the inverse of a micelle. In this system the oxidizability of DLPC is two to three times higher than that which occurs when it is homogeneously dispersed in a solvent such as *tert*-butyl alcohol. Barclay and colleagues[47] postulated that in a reverse micelle the linoleate chains on each phospholipid are "lined up" with each other as well as with the linoleate chains on adjacent molecules. Because of this proximity, the rate constant for the propagation step (k_p in Figure 19.1) is increased, causing greater oxidizability, defined earlier as $(k_p/2k_t)^{1/2}$, than that which occurs in a homogeneous system where the molecules are randomly dispersed. On the other hand, the oxidizability of DLPC in a bilayer is similar to that in a homogeneous solution. In a bilayer the peroxyl radicals, because of their greater polarity, diffuse away from the nonpolar hydrocarbon-like environment of the phospholipid acyl chains to the more polar environment of the bilayer surface. This causes a decrease in k_p. In addition, k_t also decreases. Barclay and colleagues[49] argue that this reduction in k_t could be due to stabilization of the polar peroxyl radical by the polar aqueous buffer and hydrogen bonding. Because both the numerator and denominator of the expression for oxidizability are decreased, the net change in oxidizability does not differ markedly from the oxidizability obtained for a homogeneous system.

Others have also found that if the cellular concentrations of EPA and DHA are increased by incubation *in vitro*, an increase in oxidizability occurs. Crosby and colleagues[50] measured production of conjugated dienes in human vascular endothelial cells (HUVEC) enriched with oleic acid, linoleic acid, arachidonic acid, EPA, and DHA. As cellular concentrations of EPA, DHA, and linoleic acid but not oleic acid increased, the production of conjugated dienes increased. However, the increase in conjugated dienes produced by linoleic acid was not as great as that produced by the same concentrations of EPA and DHA. Surprisingly, lipid peroxidation was decreased in the presence of an increased concentration of arachidonic acid. Using cultured rat hepatocytes exposed to various fatty acids, Sugihara and colleagues[51] found the greatest amount of the MDA:TBA_2 adduct in the cells incubated with DHA and EPA.[51]

2. Influence of Fatty Acid Composition on *in vivo* Lipid Peroxidation

Numerous studies have concluded that feeding n-3 fatty acids to animals or humans increased lipid peroxidation. Draper and colleagues[52] and Dhanakoti and Draper[53] found increased MDA:TBA_2 adduct in urine of rats fed for 9 to 10 weeks diets that contained cod liver oil compared with rats fed the same diet but replacing the cod liver oil with corn oil. Odeleye and colleagues[54] saw an increase in exhaled ethane, hepatic conjugated dienes, and lipid fluorescence in rats fed diets that contained 4% by weight cod liver oil compared to feeding the same amount of fat as olive oil, corn oil, or cottonseed oil. L'Abbe et al.[55] fed rats diets that were 20% fat by weight, with menhaden oil comprising 25 or 50% of the fat, for 16 weeks and measured increased urinary, heart, and liver TBARS compared with the values measured in rats fed the same amount of fat with 60% lard and 40% corn oil. Skuladottir et al.[33] fed rats 20% fat diets which were more than three quarters fish oil or no fish oil for 4 weeks and measured lipid hydroperoxides by adapting the methylene blue method for tissue homogenates. The animals fed the n-3 fatty acids had significantly higher levels in liver and heart. Wander and colleagues[56] fed healthy female geriatric beagle dogs diets in which the ratio of n-6 to n-3 fatty acids was 31:1, 5.4:1, and 1.4:1. They found that TBARS was higher in both plasma and urine in the dogs fed the diets that contained the greatest amounts of n-3 fatty acids. Javouhey-Donzel and colleagues[57] found the endogenous levels of TBARS and the levels induced with superoxide radicals were higher in rats fed long-chain n-3 fatty acids.

Fewer studies have been conducted in humans to evaluate the influence of the consumption of EPA and DHA on *in vivo* indices of lipid peroxidation. Meydani and colleagues[58] found significantly higher plasma concentration of TBARS in women who took six capsules of n-3 fatty acid fish oil concentrate, providing about 9 g of EPA and DHA, daily for 3 months compared with baseline values. Nelson and colleagues[59] fed nine healthy men diets that contained approximately 2% of their calories

as long-chain n-3 PUFA from salmon and measured an increase in urinary TBARS and MDA:TBA_2 adduct after the salmon diets were consumed. However, it could not be determined in this study if the increase in urinary MDA was a consequence of an increased intake of oxidized lipids that resulted when the salmon was cooked[60] or the result of *in vivo* oxidation. Wander and colleagues[61,62] gave postmenopausal women 15 g supplements of EPA/DHA-rich fish oil, providing 4.3 g of EPA and DHA, for 5 weeks. Urinary excretion of TBARS and MDA:TBA_2 adduct and plasma TBARS and MDA:TBA_2 adduct increased. This increase did not appear to be a result of the increased intake of oxidized lipids. The fish oil was obtained from the National Institute of Health Fish Oil Test Material Program and contained minimal lipid peroxides at the beginning of the study. Measures of oxidation of the oil, peroxide value and anisidine value, did not increase during the study or under conditions that simulated the manner in which the subjects would handle the supplement.

3. Confounding Factors in the Measures of Increased Oxidation of EPA/DHA-Enriched Biological Systems

There are several issues of concern in these studies. A common theme to the bulk of this work is that lipid peroxidation was assessed by measuring MDA, either as TBARS or the MDA-TBA_2 adduct. Therefore, the results must be interpreted with caution.[16,21] To use it as the sole basis for concluding that increased lipid oxidation has occurred in an *in vivo* system is likely an overinterpretation of the data.

In the animal studies the amount of n-3 fatty acids fed has little relevance to the human diet. It is common to provide 10% of the weight of the animal diets as fish oil, an amount which could easily mean that a diet was 1.5% EPA and DHA. It is difficult for humans to obtain more than 5 g of EPA and DHA from reasonable amounts of a fatty fish such as salmon.[63] This represents less than 0.5% of the diet by weight. Although the amount of EPA and DHA given to the subjects by Wander and colleagues[63] could be found in large servings of fatty fish, the amounts given to their subjects by Meydani and colleagues,[58] Nelson and colleagues,[59] and Haglund and colleagues[64] are much larger than could reasonably be found in the usual American diet. However, it must be recognized that although these large intakes could not reasonably be obtained from a diet, they could be consumed by the use of fish oil supplements.

A serious concern, especially in some of the older studies using fish oil, is that increased concentrations of lipid peroxide products measured *in vivo* may be derived from increased ingestion of them; i.e., lipid peroxidation may have occurred *in vitro* rather than *in vivo*. In 1992, Gonzales and colleagues[65] recommended that animal diets containing fish oil as their fat base be handled with extreme caution, including mixing diets in inert atmospheres, storing diets at –20°C, and providing fresh diet every 24 h in clean food jars.[65] Fritsche and Johnston[66] also reported oxidation of purified diets that contained fish oil when exposed to air at room temperature, and Tsai and colleagues[67] pointed out the importance of light and temperature in oxidation of these oils in semipurified diets. Shukla and Perkins[68] found oxidative polymeric materials in fish oil capsules. It had previously been reported that soft gelatin capsules were permeable to oxygen.[69] However, the overall importance of increased oxidation in the diet or in supplements is not clear because oxidized lipids are poorly absorbed.[70]

The studies discussed above illustrate the kinds of studies that have occurred over the last 20 years to evaluate increased oxidation in biological systems enriched in EPA and DHA. Because of the numerous issues raised suggesting design flaws, the conclusion that EPA/DHA enrichment of biological systems increases lipid peroxidation remains in question.

B. Decreased Oxidation in EPA/DHA-Enriched Biological Systems

In contrast to studies suggesting that lipid peroxidation increases in EPA/DHA-enriched biological systems, numerous studies have concluded the contrary. An intriguing array of experiments

conducted in dispersions of fatty acids in aqueous solutions to simulate biologicals systems reported that long-chain n-3 fatty acids have greater oxidative stability than n-6 fatty acids.[71–74] Free fatty acids when dispersed in water form micelles that can be stabilized by an emulsifier, i.e., one of the numerous polyoxyethylene sorbitan monoesters (Tweens) or sodium dodecyl sulfate (SDS) such as Triton-X.

Using an aqueous dispersion of methyl or ethyl esters of individual fatty acids stabilized with Tween 20, Miyashita and colleagues[75] monitored the loss of fatty acids by GC. They found that when autoxidation was initiated by hydroxyl radicals generated by ferrous sulfate and ascorbic acid in micelles containing individual fatty acids, loss of DHA was the slowest, followed by EPA, arachidonic acid, α-linolenic acid, and γ-linolenic acid (18:3n-6). Linoleic acid was the most susceptible fatty acid to oxidize. This sequence of fatty acid oxidizability was entirely opposite to what was observed in homogeneous solutions. Loss of fatty acids was also monitored in micelles that contained both linoleic acid and DHA. The more DHA that was present, the slower the loss of the total amount of substrate (the sum of linoleic acid and DHA). However, when the loss of the individual fatty acids was monitored, DHA disappeared more rapidly than did linoleic acid. Using different initiators of peroxidation, photoirradiation, and the water-soluble azo initiator 2,2′-azo-bis(2-amidinopropane) dihydrochloride (AAPH), respectively, Bruna and colleagues[71] and Visioli and colleagues[74] found that loss of EPA and DHA from micelles was slower than the loss of linoleic acid from micelles. Visioli and colleagues[74] also noted that the rate of formation of conjugated dienes was greater for linoleic acid than either EPA or DHA.

Yazu and colleagues[76] also measured the kinetics of long-chain n-3 fatty acid oxidation in chemical systems as well as micelles by monitoring both oxygen uptake using an oxygen electrode and consumption of EPA and linoleic acid by GC. Both AAPH and a lipid-soluble azo-initiator, AMVN, were used to initiate lipid peroxidation. In chlorobenzene containing only EPA, the rate of loss of EPA was greater than that of linoleic acid in a chlorobenzene solution. However, when Triton-X stabilized emulsions were prepared from each of the fatty acids separately, linoleic acid loss was faster. In addition, in a 1:1 aqueous dispersion of EPA and linoleic acid, the rate of total substrate (EPA and linoleic acid) disappearance was reduced by a factor of five compared with the rate of loss when linoleic acid was oxidized alone. However, similar to the observations made by Miyashita and colleagues,[75] the rate of disappearance of EPA in this system was faster than that of linoleic acid. Yazu and colleagues[76] argue that the peroxyl radicals derived from EPA are more polar than those derived from linoleic acid. They contend that the increase in polarity stems from the fact that the EPA-derived peroxyl radicals contain two molecules of absorbed oxygen rather than one as the linoleic acid peroxyl radicals do, a reasonable assumption in view of the known propensity for EPA to form cyclic peroxides by internal abstraction of a hydrogen atom from a nearby *bis*-allylic hydrogen.[26] In support of this notion, they measured about twice as much oxygen taken up by oxidation of EPA micelle compared to oxidation of the linoleic acid micelle. They further argue that because of their greater polarity, the EPA peroxyl radicals diffuse from the internal lipophilic region to the polar surface of the micelles. Once at the surface, because of the proximity of other radicals that also diffused to the area or initiator-derived radicals, the termination reactions for the EPA-derived peroxyl radicals increased. In effect, the EPA-derived peroxyl radicals removed the radicals necessary for chain propagation. Yazu and colleagues[76] contend that this conjecture is confirmed by the fact that the total rate of substrate disappearance in the EPA/linoleic acid micelles is reduced.

Later Yazu and colleagues[73] substantiated their hypothesis about location of EPA-derived peroxy radicals by making Triton-X micelles containing EPA or linoleic acid with antioxidants that locate at different sites in the micelle. They used BHT, which locates in the core of the micelle[77,78]; 2,2,3,7,8-pentamethyl-6-chromanol (PMC), which locates at its surface[79]; and 2,5,7,8-tetramethyl-6-chromanol (Trolox), which locates in the aqueous phase.[80] By noting which antioxidant had the greatest effect on the loss of EPA and linoleic acid, they inferred the location of their respective peroxyl radicals. When oxidation of linoleic acid was initiated with the water-soluble AAPH in the presence of each of these antioxidants, the rate of oxygen uptake was retarded; i.e., the induction

period increased. The induction period was longest with BHT, followed by PMC, and Trolox in decreasing order. This strongly suggests that the most effective protection of linoleic acid from oxidation occurred in the microenvironment that contained both the linoleic acid–derived peroxyl radical and BHT, the internal core. On the other hand, the induction period for EPA was not inhibited to any great extent by BHT compared to the inhibition that occurred in the presence of PMC and Trolox. This observation supports the argument that the EPA radicals are located at the surface of the micelle, thereby having access to the protective effects of PMC and Trolox.

The primary limitation of these studies to understanding *in vivo* oxidation is that they were conducted in micelles stabilized with emulsifiers. Although micelles are structurally less complicated than biological membranes, they may offer a good model for studying lipoproteins. In any event, they are superior to homogeneous systems as a model for they allow for the measurement of oxidation indices in systems that have discrete phases.

A number of studies conducted *in vitro*, *ex vivo*, and *in vivo* rather than in simulated systems also have found that less oxidation occurs in the presence of EPA and DHA. Vossen and colleagues[81] modified the fatty acid composition of HUVEC by culturing the cells in media supplemented with different fatty acids. They then extracted the phospholipids from the cells and made liposomes. They measured peroxidation induced by copper and hydrogen peroxide by monitoring the loss of fatty acids and the formation of conjugated dienes. The loss of EPA and DHA from the liposomes was greater than that of the less-unsaturated fatty acids. However, the formation of conjugated dienes was greatest in the liposomes with the greatest amount of linoleic acid. Calviello and colleagues[82] obtained red blood cells from rats given n-3 fatty acids and subjected them to oxidation with *tert*-butylhydroperoxide. The red blood cells from the rats given EPA and DHA were not more susceptible to oxidative damage than those obtained from rats given oleic acid as indicated by MDA release into the medium or hemolysis. Burns and colleagues[83] found that endogenous concentration of TBARS was not changed in lungs and brains from mice fed diets that contained 10% sardine oil, providing about 3 g EPA and DHA per 100 g diet. However, *in vitro* production of TBARS by lung tissue was greater in the mice fed fish oil than those fed sunflower oil or beef tallow. Lamers and colleagues[84] found similar levels of TBARS in pigs fed either lard– or fish oil–based diets for 8 weeks. Demoz and colleagues[85] found that TBARS concentrations were actually lower in mice administered purified EPA by gastric intubation daily for 10 days compared with those given palmitic acid.

Similar, but fewer, observations have been made in humans. Hansen and colleagues[86] gave healthy male volunteers 4 g capsules of purified esters of EPA or DHA. Neither changed the plasma concentration of TBARS. Higdon and colleagues[87] gave 15 healthy postmenopausal women 15-g supplements of fish oil supplying approximately 3.4 g of EPA and DHA daily, safflower oil supplying 10.5 g of linoleic acid, and sunflower oil providing 12.3 g of oleic acid in a crossover design for 5 weeks and estimated plasma lipid peroxidation using three different measures. The concentration of plasma TBARS was highest after supplementation with fish oil and similar after supplementation with safflower oil and sunflower oil. However, the concentration of F_2-isoprostanes and MDA measured by GC/MS was lowest after supplementation with fish oil. The higher values measured by TBARS may reflect the nonspecificity and artifactual increases of this assay. The lower value obtained by measuring F_2-isoprostanes is confounded by the fact that consumption of the fish oil simultaneously lowered plasma concentration of arachidonic acid. Measurement of MDA by GC/MS may accurately indicate *in vivo* oxidation because it is a highly specific, sensitive assay.

Proteins can also be changed by oxidants.[16] Few studies have addressed increased protein damage that may occur when EPA and DHA are consumed. Although several methods exist for measuring protein damage, the carbonyl assay is considered a general assay of oxidative damage to proteins.[88] Wander and Du[61] used the production of protein carbonyls as a measure of lipid peroxidation in 48 postmenopausal women given 2.5 g of EPA and 1.8 g of DHA daily for 5 weeks. No difference was found in the amount of plasma protein carbonyls produced after supplementation compared with before supplementation.

Collectively, these experiments suggest that the degree of total oxidation that occurs in biological systems enriched with long-chain PUFA may not be explained solely by their degree of unsaturation. They support the necessity of understanding not only the method used to assess the extent of lipid peroxidation but also the role of discrete phases in complex systems to understand the oxidizability of fatty acids.

VI. OXIDATION OF EPA/DHA-ENRICHED LDL

The oxidative susceptibility of LDL enriched with specific fatty acids deserves special scrutiny since oxidized LDL (oxLDL) may play a significant role in the formation of atherosclerotic lesions.[89] LDL enriched with oleic acid has been shown in several studies to be less susceptible to oxidization than PUFA.[27,90,91] Oxygen consumption is less,[27] lag time is longer, the propagation rate is slower, and the amount of conjugated dienes formed is smaller after consumption of the diets rich in oleic acid.[90,91] Lee and colleagues[92] have even shown that liposomes enriched with oleic acid have less proinflammatory activity.

The effect of enriching LDL particles with EPA and DHA on their oxidative susceptibility have produced more confusing results. In a porcine model of atherosclerosis,[93] LDL from fish oil–fed animals oxidized more readily. However, no difference was observed in lesion regression between these animals and those given no oil supplement. Tsai and Lu[94] found that fish oil consumption shortened the lag time for the production of conjugated dienes and increased the production of TBARS in LDL from healthy males. Hornstra and coinvestigators[95] gave seven healthy male volunteers 2 g of n-3 fatty acids per day for 3 weeks. They observed a shorter lag time but no difference in the total amount of conjugated dienes formed or the rate at which they were formed in the group given fish oil compared with four men who received no supplement. Harats and co-workers[96] measured the effect of a 10-g supplement of fish oil concentrate taken daily for 4 weeks on LDL *ex vivo* oxidative susceptibility in smokers and nonsmokers. Both groups had significantly higher LDL TBARS after the ingestion of the fish oil supplement, but the values were higher in smokers.

On the other hand, several studies have shown that the effect of enriching LDL with EPA and DHA on its oxidative susceptibility cannot be clearly interpreted. Wander and colleagues[62] gave 48 postmenopausal women 15-g supplements of fish oil daily for 5 weeks. Lag time was shortened 18 min, but, paradoxically, the rate of formation of conjugated dienes was 25% slower. Sorensen and colleagues[97] made similar observations in LDL from subjects given either a sunflower oil or fish oil margarine for 4 weeks. Brude and colleagues[98] gave 41 male smokers 5-g supplements of EPA/DHA daily in a randomized, double-blind, placebo-controlled trial. EPA/DHA-enriched LDL oxidative susceptibility initiated by cuprous ions, AAPH, or autologous mononuclear cells was similar to that of LDL obtained from the sunflower oil–fed men except for the rate of formation of conjugated dienes, which was slower in the EPA/DHA-enriched LDL. Wander and colleagues[99–101] gave 15 postmenopausal women supplements of EPA/DHA-rich fish oil, linoleic acid–rich safflower oil, and oleic acid–rich sunflower oil in a three-way crossover design for 5 weeks. The safflower oil supplement significantly increased the concentration of linoleic acid, the sunflower oil supplement that of oleic acid, and the fish oil that of EPA and DHA in LDL. The lag time was shortest in the total fraction of LDL, as well as in the light and heavy fractions after the consumption of the fish oil supplement, and longest after the sunflower oil supplement. The rate of formation of conjugated dienes was slowest after the fish oil supplement and fastest after the safflower oil supplement in both the total fraction and the two subfractions of LDL. In addition, the formation of the lipid hydroperoxides from cholesteryl linoleate in the LDL core and phospholipid peroxides from the LDL surface was measured by postcolumn chemiluminescence. Although the lag time was shorter, the rate of formation of lipid hydroperoxides and the total amount formed was less.[101] Barbeau and colleagues[102] investigated LDL oxidation in female Yucatan miniature swine fed an atherogenic diet supplemented with either fish oil or a mixture of corn oil, safflower

oil, and beef tallow. They too found that fish oil–enriched LDL had a 30% shorter lag time and a 30% slower oxidation rate but no difference in atherosclerotic lesion development of all major blood vessels. Thomas and colleagues[103] measured rates of oxidation of LDL isolated from cynomolgus monkeys and found that linoleate-enriched diets gave significantly higher rates than saturated, monounsaturated, or EPA/DHA-enriched LDL. Saito and colleagues,[104] Nenseter and coworkers,[105] and Frankel and colleagues[106] also noted that fish oil consumption did not alter oxidative susceptibility of LDL. Ramirez-Tortosa et al.[107] found that healthy male patients consuming either olive oil and fish oil or olive oil alone had similar rates of formation of conjugated dienes but the oxLDL from subjects given both supplements was less readily taken up by macrophages.

Although the design of these studies differs, i.e., in amount of supplement given, duration of supplementation, and species, they collectively show that the oxidative susceptibility of LDL enriched with EPA and DHA is not well understood. The more relevant question, however, is if indeed oxLDL contributes to the development of atherosclerotic plaque, does enriching the LDL particle with EPA and DHA change its impact on plaque development?

VII. IS THE REQUIREMENT FOR VITAMIN E INCREASED WHEN EPA AND DHA INTAKE INCREASES?

Despite the fact that contradictory arguments exist about susceptibility of EPA/DHA-enriched biological systems to oxidize, the prevailing opinion is that more vitamin E, particularly α-tocopherol, should be consumed as more PUFA are eaten to prevent increased lipid oxidation. When the primary dietary PUFA is linoleic acid, a ratio of 0.4 mg α-tocopherol to 1.0 g PUFA is considered appropriate for humans.[108] Witting and colleagues[109] investigated the amount of tocopherol necessary to protect fatty acids of different degrees of unsaturation from oxidation by studying the rate of the development of creatinuria in rats, a sign of tocopherol deficiency. Using their data, Muggli[110] calculated that for humans 1.5 mg RRR-α-tocopherol should be added to the diet for every gram of EPA consumed and 1.8 mg for every gram of DHA.

Studies to support the necessity of increased intakes of vitamin E when EPA and DHA are consumed have produced remarkably different results (Table 19.1). Comparisons among the various studies are hampered by the fact that they have been conducted in numerous species (rat, mouse, dog and humans) with different doses and forms of vitamin E, sources of EPA and DHA, background diets, and duration of intervention. Vitamin E status, as indicated by the concentration of α-tocopherol in tissues and plasma, and extent of peroxidation, have been used as end points. Many of the studies in rodents,[111–115] but not all,[116,117] have shown that α-tocopherol expressed on a volume basis is decreased in plasma when EPA and DHA are consumed. This is frequently interpreted to mean that EPA/DHA consumption induces oxidative stress and, as a consequence, α-tocopherol concentration is lowered. However, if the data are expressed normalized to the lipid content of plasma, since EPA/DHA consumption consistently lowers the concentration of plasma lipids, the differences in α-tocopherol concentration of plasma between non-EPA/DHA- and EPA/DHA-supplemented animals often disappear.[111,117,118] Some tissues, e.g., liver, kidney, and lung, may also demonstrate a lower concentration of α-tocopherol when EPA/DHA is consumed. However, in other tissues, e.g., immune cells (splenocytes and thymocytes)[117] and heart,[111] the levels increase. Vitamin E concentration in plasma and tissues increases in rats[113,114,116,119] and dogs[118] as dietary levels increase. However, even when the intake is more than 20 times the required amounts, plasma concentration expressed on a volume basis remains lower in dogs given EPA/DHA compared with dogs not given these fatty acids, but are equivalent expressed on the basis of plasma lipids.[118]

In contrast, studies conducted in free-living populations of humans that addressed the influence of the consumption of EPA/DHA on plasma vitamin E status have shown that plasma concentration of α-tocopherol expressed on volume basis increased[115,120] or did not change[58,62,87] relative to diets not enriched in EPA/DHA. Only in a metabolic feeding study where diets where

TABLE 19.1
Summary of Studies Showing the Influence of Consumption of EPA and DHA on Vitamin E Status in Different Species and Tissues

Study Design	Observations	Ref.
Species: Rat		
Diets contained 17% fat (w/w); n-6/n-3 = 51,4.6,2.0,0.8; duration: 4 wk; 200 mg αT and αTA[2]/kg diet	At n-6/n-3 = 4.6, 2.0, 0.8; *Plasma:* TG ↓, C ↓, αT/V ↓, αT/L ↔[3] *Liver:* αT/g ↓[4] , αT/L ↓; *Heart:* αT/g ↑↑, αT/L ↑↑	111
Diets contained 20% fat (w/w); L = control; MFO and SFO supplied similar amounts of EPA/DHA[5]; duration: 10 wk; 30 mg αT/kg diet	*Plasma:* αT/V and αT/L ↔ with MFO, SFO *Liver:* αT/g ↓, αT/L ↔ with MFO, SFO *Immune cells:* ↑ with MFO, SFO	117
Diets contained 20% fat (w/w); L = control; MFO and SFO supplied similar amounts of EPA/DHA; 60 mg αT and αTA/kg diet	*Plasma:* αT/V ↓ with MFO, SFO *Liver:* αT/g ↓ with MFO, SFO *Immune cells:* αT ↔ if form of E controlled	112
Diets contained 10% fat (w/w); CO[6] and MFO; duration: 5 wk; ≤5, 30, 150 vitamin E/kg diet	*Liver and kidney:* TBARS and CD[7] ↓ with increasing E with both oils	126
Diets contained 10% fat (w/w); EXPT 1: DHA at 0, 1.0, 3.4, 8.7 en%; EXPT 2: DHA at 8.7 en%; duration = 14 d; EXPT 1: 134 mg αTE[8]/kg diet; EXPT 2: 54, 134, 402 mg TE/kg diet	EXPT 1- TBARS ↑ in serum, liver, kidney and chemiluminescence ↑ in liver at 3.4, 8.7 en% DHA; αT↓ as DHA↑ EXPT 2-liver and kidney: αT ↑ and TBARS ↔ in vitamin E groups	113
Diets contained 20% fat (w/w); control = high MUFA or 'average' 18:2n-6; Trt = fish oil, linseed oil, sunflower oil; duration = 11 wk; none, 41, 82, 124 mg all-*rac*-αTA/kg diet + matched RRR-α-T and γT	*Serum:* αT/V↓on all trt diets compared to control and ↑ with dietary αT except with FO *Liver:* αT↓on all trt diets compared to control and ↑ with dietary αT	114
Diets contained 10% fat (w/w); control = SO[9]; trt = MFO; duration = 8 wk, ≤3, 45, 209 mg RRR-α-TAkg/diet; SO only 45 mg/kg diet	*Plasma:* TL[10]↓ , αT/V ↑, TBARS ↓ with α-TA in trt groups; $\alpha T/L_{MFO} > \alpha T/L_{SO}$; $TBARS/LM_{FO} > TBARS/L_{SO}$ *Liver:* αT/g ↑ with α-TA in trt groups; $\alpha T/g_{MFO} > \alpha T/L_{SO}$; $TBARS_{MFO} > TBARS_{SO}$	116
Species: Mouse		
Diets contained 5% fat (w/w); MFO, CO, coconut oil; 30, 100, 500 mg/kg RRR-α-TA/kg diet	*Plasma:* αT/V = MFO < CO < coconut oil *Liver, kidney, lung:* αT/g = MFO < CO < coconut oil	115
Diets contained 10% fat (w/w); FO[11] and L; duration = 4 wk; vitamin E deficient or 1 g all-*rac*-α-TA/kg diet	*Liver:* TBARS, CD, protein carbonyls ↓ in FO with E but not L; αT/L ↑ with E in FO and L	119
Species: Dogs		
Diets contained 5% fat (w/w) with n-6/n-3 = 40:1 and 1.3:1; αTA = 17, 106, 446 mg/kg diet	*Plasma:* αT/V ↓, αT/L ↔, TBARS ↑ with 1.3:1; αT/V, αT/L ↑, TBARS ↑ as dietary αTA ↑ but more in 40:1 diet	118
Species: Humans		
12 ♀ and ♂; supplement = 2 kinds FO, one with 5 mg αT, one with 20 mg αT; free-living	*Plasma:* MDA:TBA_2 ↑ with low αT	64

continued

TABLE 19.1 (CONTINUED)
Summary of Studies Showing the Influence of Consumption of EPA and DHA on Vitamin E Status in Different Species and Tissues

80♂; diets contained 30–40 en% fat; supplement = 6.3 g EPA/DHA or 6.1 g MUFA from ethyl esters; free-living; duration = 6 wk; no vitamin E or 900 mg all-*rac*-αTA with each diet	*Plasma:* αT/V higher after E but similar with both fat supplements; TBARS and methylene blue ↑ n-3 groups but not changed by E; breath hydrocarbons ↔ in all groups	125
10♂; diets contained 38 en% fat; supplement = 18 g FO (4.6 g EPA/DHA); duration = 6 wk; 10 αTE from diet; 24 mg T from supplement; free-living	*Plasma:* αT/V ↑ with FO	120
25♀; diets contained 35–40 en% fat; 6 g FO (2.4 g EPA/DHA); duration = 2 mo; 4 mg RRR-αT from supplement; free living	*Plasma:* TG ↓, αT/V ↔, αT/L ↑, TBARS ↑ with FO	58
40♂; diets contained 40 en% fat; supplement = 15 g placebo or FO (7 g EPA/DHA);15 mg all-*rac*-αT in FO; no E supplement for 1st 10 wk; 200 mg αT/d last 8 wk; metabolic feeding study	*Plasma:* TBARS ↑ with FO but ↓ with E + FO *Plasma, platelets, RBC:* αT ↓ but ↑ with E + FO	121
48♀; diets contained 30 en% fat; supplement = 15 g FO (4.3 g EPA/DHA); 0, 100, 200, 400 mg αTA; free-living	*Plasma:* αT/V and αT/L ↔ with FO but ↑ with E; TBARS and MDA:TBA_2 ↑ but not protein carbonyls with FO, but not changed with E *Urine:* TBARS and MDA:TBA_2 ↑with FO; only urine TBARS ↓ with E	61,62

[1] Total dietary fat and measure of n-3 fatty acid content of diet.
[2] T = α-tocopherol; α-TA = α-tocopheryl acetate; stereoisomer is identified if known.
[3] TG = triacylglycerol; C = cholesterol; αT/V = α-tocopherol relative to volume; α-tocopherol/L = α-tocopherol relative to lipids; ↑ = higher with indicated dietary manipulation; ↓ = lower with indicated dietary manipulation; ↔ = no change with indicated dietary manipulation.
[4] αT/g = α-tocopherol relative to tissue weight.
[5] L = lard; MFO = menhaden fish oil; SFO = sardine fish oil; CLO = cod liver oil.
[6] CO = corn oil.
[7] CD = conjugated dienes.
[8] TE = α-tocopherol equivalents.
[9] SO = soybean oil.
[10] TL = total lipids.
[11] FO = fish oil.

controlled[121] and in a study in which plasma concentrations were compared in women supplemented with an oleic acid–rich oil to the same women supplemented with fish oil[87] did plasma α-tocopherol concentrations decrease. Normalizing the α-tocopherol concentration of plasma to its lipid content often changes the conclusions drawn about its status. In the study conducted by Meydani and colleagues[58] in women, plasma α-tocopherol concentration relative to lipid increased while on a volume basis it was unchanged when fish oil was consumed, whereas Higdon and colleagues[87] reported that the differences noted between supplementing with fish oil compared with sunflower oil were obliterated. When vitamin E supplements are administered concurrently with EPA/DHA, the plasma concentration of α-tocopherol increased regardless of how the data were expressed[61,121] to levels that are similar to those measured in subjects given similar amounts of vitamin E but no EPA/DHA.[122–124]

When adequacy of vitamin E intake is assessed by monitoring the extent of lipid peroxidation, the conclusions drawn from studies in both animals and humans depend upon the manner is which lipid peroxidation was measured. In the main, EPA/DHA consumption has been shown to increase plasma TBARS concentration.[58,61,87,116,121,125] This increase may[125] or may not[61] be lowered by vitamin E supplementation. When other more specific measure of lipid peroxidation are used, e.g., conjugated dienes and total volatiles,[126] breath alkane,[125] protein carbonyls,[61] F_2-isoprostanes,[87] MDA by GC/MS,[87]or MDA:TBA_2 in urine and plasma,[62] it is less clear that EPA/DHA consumption poses an oxidative stress, as discussed earlier. Moreover, in those instances where an increase in any of these variables occurs, vitamin E supplementation appears to be of limited usefulness to decrease it.[62,125]

Collectively these studies clearly illustrate that effect of the consumption of fish or fish oil on vitamin E homeostasis remains unanswered. The conclusions drawn depend upon how data are expressed, the tissue evaluated, and the measure of lipid peroxidation used. As the presumed increased in lipid peroxidation that occurs in the face of increased consumption of EPA and DHA forms part of the data on which the recommended dietary intake of vitamin E is based,[108] this issue needs more definitive studies so that it can be resolved.

VIII. POSSIBLE MECHANISMS FOR THE OXIDATION OF EPA AND DHA IN BIOLOGICAL SYSTEMS

EPA and DHA may oxidize quite differently in biological systems, which have discrete polar and nonpolar microenvironments, than they do in homogeneous chemical systems. A mechanism is proposed in which the rapid oxidation that occurs to EPA and DHA compared with other fatty acids in homogeneous systems also occurs in heterogenous systems. However, a different fate is proposed for the free radicals that are formed. This mechanism is discussed below in the context of oxidation of LDL.

In homogeneous systems EPA and DHA are randomly dispersed. In this environment they oxidize more readily than fatty acids with fewer *bis*-allylic hydrogen atoms and can form, in addition to hydroperoxide radicals, polar cyclic radicals such as bicycloendoperoxide radicals. In LDL, EPA and DHA are found esterified either to the phospholipids at the particle surface or the cholesterol in its core. Thus, they are present in distinct, separate phases or microenvironments. At either location they oxidize more readily than the surrounding fatty acids and form, in addition to hydroperoxides, the same cyclic radicals that are formed in homogeneous systems. The presence of more than one oxygen molecule on the radicals derived from EPA and DHA increases their polarity. Because of their greater polarity, they either remain in the polar environment of the surface phospholipids or migrate to this area from the oily core. Clusters of these more polar free radicals are formed at the lipoprotein surface. Because of their proximity, termination reactions between them are frequent. In addition, because of their location at the LDL surface, they may react with aqueous peroxyl radicals (assuming for the sake of this discussion that the oxidant is an aqueous peroxyl radical such as would be generated by AAPH) that would otherwise initiate lipid peroxidation. In either event, EPA and DHA remove free radicals from the oxidizing environment and terminate the chain reaction.

This mechanism offers an explanation for the paradoxical effect found in LDL enriched with EPA and DHA in which both the lag time and the rate of formation of conjugated dienes, phospholipid hydroperoxides, and cholesterol ester hydroperoxides are decreased.[29] Dr. Roland Stocker's laboratory at the Heart Research Institute in Australia has recently developed a model to explain LDL oxidation, termed tocopherol-mediated peroxidation (TMP).[127] In this model, again assuming that the oxidant is an aqueous peroxyl radical, the fate of α-tocopherol depends upon the frequency with which it encounters a peroxyl radical. Under conditions it which it frequently encounters a peroxyl radical, i.e., high radical flux, α-tocopherol functions in its well-acknowledged role as an antioxidant. However, under conditions in which it infrequently encounters a peroxyl radical, i.e., low radical flux, it functions as a pro-oxidant.

The rate constant for the reaction of an aqueous peroxyl radical with α-tocopherol is much greater than it is for the reaction of the peroxyl radical with a PUFA, primarily linoleic acid, found in the surface phospholipids.[16] Thus, even though the phospholipid fatty acids are present in greater abundance than α-tocopherol in LDL, the aqueous peroxyl radicals preferentially react with α-tocopherol forming an α-tocopherol radical. Under conditions of low radical flux, the relatively stable but less polar α-tocopherol radical moves within the particle to where it has access to PUFA in both the surface and core of LDL. In this way, α-tocopherol transfers radicals from the outside of LDL to the inside, an activity referred to as the phase transfer activity of α-tocopherol. In the absence of other radicals, the α-tocopherol radical can react with *bis*-allylic hydrogens on PUFA to form a carbon-centered free radical. This, in turn, reacts rapidly with oxygen to make a lipid peroxide radical which can react with α-tocopherol to regenerate the α-tocopherol radical and the process is repeated. α-Tocopherol propagates the chain reaction, a process referred to as the chain transfer activity of α-tocopherol in TMP. Most studies that have addressed the oxidative susceptibility of LDL have used high radical flux. Under these conditions, because the number of peroxyl radicals in the aqueous environment surrounding the LDL particle is high, the probability of the α-tocopherol radical encountering a radical is also high. Thus, the potentially damaging α-tocopherol radical is removed from the LDL environment before it can propagate a chain reaction. In this situation α-tocopherol prevents lipid peroxidation and in the process is rapidly depleted from the LDL particle.

This model must be modified slightly to explain the oxidation of LDL after consumption of EPA and DHA. Prior to their consumption, the surface phospholipids contain linoleic acid:EPA:DHA in a molar ratio of 41:5:1, while in the core cholesterol esters, the ratio is 55:1:1; after their consumption, the ratios become 3:1:1 and 34:5:1, respectively.[29] As a consequence of this enrichment, the ratio of DHA and EPA to α-tocopherol in the LDL particle is increased.

In EPA/DHA-enriched LDL under conditions of high radical flux, the lag time is shortened and the rate of formation of oxidized products lower. It is proposed that this occurs in the following way. Even though α-tocopherol reacts with the aqueous peroxyl radicals more rapidly than do EPA and DHA,[16] some of the peroxyl radicals will react with EPA and DHA because of their increased concentration in the LDL. The resulting radicals quickly react with two molecules of O_2 to form phospholipid and cholesterol ester peroxide radicals. These radicals rapidly consume the tocopherol radical. As a consequence, the EPA/DHA-enriched LDL particle has a shorter lag time. The rate of formation of conjugated dienes phospholipid hydroperoxides and cholesterol ester hydroperoxides will also decrease. This occurs because the EPA/DHA-derived peroxyl radicals, being somewhat polar, cluster at the surface of the LDL particle, causing termination reactions among the radicals to be more frequent. The EPA/DHA-derived peroxyl radicals also react with the peroxyl radicals at the water–lipid interface, again a termination reaction. Because the substrate is removed, the rate of formation of lipid peroxides is lower. Although the physiological significance of oxidation of LDL under high radical flux may be minimal,[128] low radical flux conditions may occur to LDL in the arterial wall.

Under low radical flux conditions, TMP propagation of LDL oxidation can be decreased in EPA/DHA-enriched LDL. The EPA/DHA will still compete effectively with α-tocopherol for the aqueous peroxyl radicals to form EPA/DHA-derived radicals. These radicals quickly react with oxygen to make peroxyl radicals. These peroxyl radicals react with the tocopheryl radical to form nonradical products preventing α-tocopherol from propagating the chain reaction. LDL oxidation is decreased by enriching LDL with EPA and DHA since the chain transfer activity of α-tocopherol is lessened.

This proposed mechanism has not been experimentally validated because most experiments to date with EPA/DHA-enriched LDL have been done under conditions of high radical flux. However, as the TMP model necessitates understanding the role of α-tocopherol in the context of the microenvironments that occur in LDL, this model requires a similar interpretation for the behavior of EPA- and DHA-derived radicals.

IX. CONCLUSION

Although the role of the long-chain fatty acids EPA and DHA on the oxidative susceptibility of biological systems is far from resolved, considerable doubt has been raised about whether they increase overall oxidation. Classical autoxidation kinetics derived from homogeneous chemical systems may not accurately represent the kinetics of heterogeneous biological systems. The role of discrete microenvironments must be examined to understand the free radical oxidation of long-chain n-3 fatty acids in biological systems.

Whatever the mechanism, a protective effect of these fatty acids toward *in vivo* oxidation may offer a partial explanation for the cardioprotective effect of fish consumption that has been shown in numerous epidemiological studies. It might also contribute to the anti-inflammatory[129] and anticancer effects[130] that these fatty acids have been reported to have. Alternatively, it abates the worry that although fish consumption may be healthy, it comes at the price of increased *in vivo* oxidation and its consequences. However, until experiments are conducted using assays that determine the differential effects of individual fatty acids on oxidation, these ideas remain speculative.

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20 Omega-3 Polyunsaturated Fatty Acids and Cardiac Arrhythmias

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I. INTRODUCTION

The beneficial role of fish oils, rich in n-3 polyunsaturated fatty acids (PUFAs) like eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), has been studied in the prevention of various human disorders like breast cancer, cardiovascular diseases (CVD), rheumatoid arthritis, and inflammatory diseases. One of the most exciting apprehensions of n-3 PUFAs is probably their ability to reduce the severe cardiac arrhythmias like ventricular fibrillation (VF). Ventricular fibrillation is a lethal cardiac arrhythmia that occurs in up to a quarter of patients suffering coronary artery occlusion with myocardial infarction. Several studies have provided evidence for the prevention of VF by n-3 PUFAs in cultured neonatal rat myocytes, in such animals as exercised dogs, rats, and marmosets, and in clinical trials. A number of mechanisms have been proposed to explain the antiarrhythmic effects of n-3 PUFAs. These include the incorporation and modification of myocytes cell membranes by n-3 PUFAs, resulting in modulation of membrane ion channels, prevention of high accumulation of intracellular calcium, production of antithrombotic eicosanoids,

0-8493-8734-5/01/$0.00+$.50
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influence on cell signaling mediated through phosphoinositides, and induction of different antioxidant enzymes. All these possible mechanisms may play an important role in antiarrhythmatic role of n-3 PUFAs in conferring cardiovascular protection. Each of these mechanisms and the studies undertaken to understand the role of n-3 PUFAs in the prevention of cardiac arryhthmias are discussed in this chapter.

II. POLYUNSATURATED FATTY ACIDS

Dietary lipids stored in adipose tissue are an important source of energy for the different processes in the body. They are also required by the body for cell structure, membrane function, and as a source of precursors for eicosanoid synthesis. Lipids are composed of fatty acids of different chain lengths and degrees of saturation as well as different configurations. There are three main classes of fatty acids: saturated fatty acids (with no double bond), monounsaturated fatty acids (one double bond), and polyunsaturated fatty acids (two to six double bonds). Polyunsaturated fatty acids are structural components of the phospholipids and glycolipids in the cellular membranes. Structurally, these are hydrocarbon chains of 20 or more carbon atoms with a carboxyl group at one end and a methyl group at the other. There are four major families of unsaturated fatty acids, n-7, n-9, n-6, and n-3 (synonymous with ω-3), where the position of the first double bond proximal to the methyl end of the fatty acid is specified.

A. Marine Sources of n-3 Fatty Acids

Omega-3 fatty acids can be obtained from marine sources or from vegetable oils in our diet. Omega-3 fatty acids of marine origin are produced in phytoplankton and algae. These are transferred via the nutrition chain and eventually incorporated into different membrane phospholipids and fat depots in fish and other marine animals, such as shrimp, seal, and whale.[1,2] In the human diet, these n-3 fatty acids are found in fatty fish (or products thereof), including herring, mackerel, salmon, trout, and in fish oil and cod liver oil.

B. Omega-3 Fatty Acids from Plant Sources

Plants can synthesize both the basic n-6 and n-3 fatty acids. Plant oils like flaxseed and canola oils have 50 and 9% of their total fatty acids as α-linolenic acid (18:3n-3). Other rich dietary sources of α-linolenic acid include soybean oil, English walnuts, green leafy vegetables, and human milk.[3] After ingestion, α-linolenic acid serves as a precursor of very long chain polyunsaturated fatty acids including eicosapentaenoic acid (20:5n-3) and docosahexaenoic acid (22:6n-3) (Figure 20.1). α-Linolenic acid may have a direct protective effect on cardiac arrhythmias, and it is likely mediated, in part, through the synthesis of EPA and DHA. The conversion of α-linolenic acid to EPA and DHA in humans is believed to be slow and competes with linolenic acid of the n-6 family.

III. CARDIAC ARRHYTHMIAS

A. Arrhythmias

Heart rhythms arise as a result of the waves of electrical excitation, which spread through the heart muscle. Individual heart cells known as cardiac myocytes are excitable and the signal, which leads to the contraction of myocytes, is the generation of an action potential at the cell membrane. Cardiac myocytes are electrically coupled to each other by gap junctions which are small pores through which electrical currents can flow from cell to cell. When a region of heart becomes ischemic, the change in electrical properties leads to abnormal heart rhythms called arrhythmias. The most

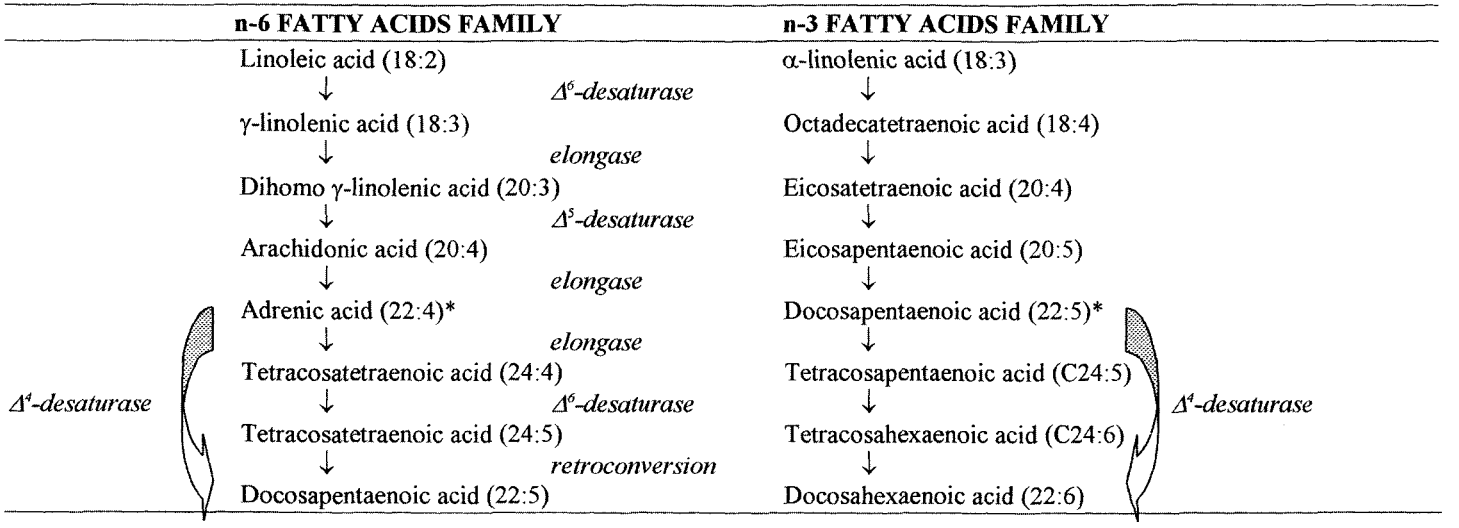

FIGURE 20.1 Formation of important n-3 and n-6 fatty acids by elongation and desaturation of the essential fatty acids linoleic acid (LA) and α-linolenic acid (α-LA). *In the old pathway, these were believed to be carried out by Δ^4-desaturase as indicated by shaded arrows.

common fatal arrhythmia is known as VF, in which electrical impulses from damaged cardiac muscle cause the normally synchronous contractions of the heart to break down.[4] It is believed that at least half of the deaths due to coronary artery disease in United States are the result of the disturbances in the electrical stability of the heart which terminates in VF.[5] In most cases, arrhythmias occur without previous symptoms and causes immediate loss of conduction and death.

B. Ischemia-Reperfusion Damage and Development of Cardiac Arrhythmias

Cardiovascular function reflects the properties of the different blood vessels of the circulatory system and of the heart, which pumps blood through these vessels. When a part of the heart loses its blood supply by occlusion of a branch of coronary artery, it loses its ability to function and this condition is termed as ischemia. If the blockage is not cleared, the cells in the affected part of the heart will undergo necrosis and die. A number of metabolic changes are set into motion in myocardium by ischemia, including cessation of contraction and alteration in membrane potential.[6] Cardiac arrhythmias occur during the early and potentially reversible phase of ischemia and after reperfusion. Reperfusion has been shown to increase oxidation in tissues.[7,8] Oxidants such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2) play an active role in host-mediated destruction of foreign pathogens but excessive production of oxidants may cause damage to the cardiovascular system in normal individuals.[9-11] Reperfusion after a prolonged period of ischemia results in glutathione (GSH) release from the myocardium, where GSH in combination with other antioxidant enzymes constitutes a significant defense mechanism against oxygen toxicity. The cellular GSH becomes a rate-limiting factor for detoxification of oxygen metabolites, most likely lipid hydroperoxides and hydrogen peroxide, resulting in oxidative stress. Oxidative stress, particularly at the mitochondria level, may also lead to programmed cell death or apoptosis.[12]

Oxidative modification of low-density lipoproteins (LDL) in the arterial intima may promote the formation of atherosclerotic lesions not only by induction of macrophage foam cell generation but also by inducing intracellular Ca^{2+} mobilization, which has been suggested to play an important role in myocardial apoptosis and smooth muscle proliferation via induction of different protein kinases and nuclear transcription factors.[13,14] A high intracellular Ca^{2+} can cause cellular damage in many different ways, including myocardial injury and cell necrosis. Oxidants may stimulate several different signal-transduction pathways, which generate second messengers. Typically, oxidants increase production of eicosanoids, inositol triphosphates (IP_3), diacylglycerol (DAG), and phosphatidic acid (PA) through stimulation of PLA_2, PLC, and PLD activities. Increase in intracellular acid generation in cardiac myocytes has also been observed during ischemia reperfusion. This may stimulate Na^+/H^+ exchange with a subsequent increase in intracellular Na^+ level. The rising intracellular Na^+ concentration then activates Ca^{2+} influx via Na^+/Ca^{2+} exchange resulting in a Ca^{2+} overload and cell death and arrhythmias.

IV. PREVENTATIVE ROLE OF N-3 FATTY ACIDS IN CARDIAC ARRYTHMIAS

Epidemiological studies have shown the prevention of ischemia-induced cardiac sudden death by n-3 PUFAs in cultured myocytes,[15,16] animals, and clinical trials. [17]

A. Epidemiological Studies

Although fish and other seafood have been a part of the diet of coastal populations for centuries, their nutritional effects were brought to attention by Bang and Dyerberg[18,19] in the late 1970s. The diet of Eskimos in Greenland and Alaska was found to be very rich in DHA and EPA and there was a direct correlation of this diet with low incidence of thrombosis and ischemic heart disease (IHD). Since then, numerous studies have covered the biochemistry and beneficial therapeutic use of n-3 fatty acids in clinical fields such as lipid disorders,[20] renal,[21,22] rheumatic,[23] cardiovascular[24-26]

and gastrointestinal disorders[27,28] and in neonatal development.[29,30] Observational studies in humans have shown that there is a connection between the intake of n-3 fatty acids and a lower risk of sudden cardiac death. Dietary intake of n-3 polyunsaturated fatty acids from seafood is associated with a reduced risk of primary cardiac arrest.[31] The first randomized controlled study, the Death and Reinfarction Trial (DART), was published in 1989.[32] In this study, 1015 men who just had a myocardial infarction were randomly advised to eat fatty fish at least twice per week or to take a modest supplement of fish oil. Another 1018 men were simply not given advice to eat fish. After 2 years, there was 29% reduction in deaths from coronary heart diseases, but there was no reduction in new cardiac events. These results suggest that the decrease in mortality resulted from reduction in sudden deaths. Later a similar reduction in cardiovascular deaths (76%) was reported in an α-linolenic acid–rich Mediterranean diet study in 1994.[33] Some trials suggest that fish oils can prevent ventricular arrhythmias in humans. The well-known effects of fish oil in reducing hypertriglyceridemia[34] and thrombosis[35,36] may partly explain this preventive effect. It is possible that the effect of fish oils on arrhythmias is independent of their antiatherogenic and antithrombotic activities. Studies conducted in Japan and Holland also provide evidence of protection against IHD by dietary intake of fish.[37] In another recent study, a placebo-controlled double-blind study assessed the effect of dietary n-3 PUFAs on the frequency of ventricular premature complexes in patients with good ventricular function who experienced frequent, nonfatal ventricular arrhythmias.[38] The patients were assigned to receive either fish oil (a total of 2.4 g n-3 PUFA/day) or sunflower seed oil as placebo for 16 weeks. They found that ventricular premature complexes decreased by 48% in the fish oil group and by 25% in the placebo group. In a more recent randomized fish trial involving 360 Indian patients with postmyocardial infarction, three randomized groups were formed: those receiving fish oil, or mustard oil, or placebo.[39] After 1 year, the mortality results were 24, 28, and 34% respectively, with a significant difference between the fish oil and placebo group ($p < 0.05$). A recent study also showed that n-3 PUFA reduced the risk of cell death from myocardial infarction and stroke in patients during the 3.5-year follow-up period.[40]

B. Experimental Animal Studies

Experimental animals like rodents (rats), nonhuman primates (marmoset monkeys), and dogs have been used to demonstrate the effects of different fatty acids on cardiac arrhythmias. Following some earlier reports[41,42] about antiarrhythmic effects of unsaturated fatty acids in animals, protective effect of dietary pure omega-3 polyunsaturated fatty acids against ischemia-induced fatal ventricular arrhythmias was observed in dogs.[43] With infusion of the eicosapentaenoic acid, five of seven dogs were protected from fatal ventricular arrhythmias ($p < 0.02$). With docosahexaenoic acid, six of eight dogs were protected, and with α-linolenic acid, six of eight dogs were also protected ($p < 0.004$ for each). Abeywardena and colleagues.[44] examined the effect of long-term dietary supplementation of different dietary fatty acids on arrhythmias in rats and marmoset monkeys. This study demonstrated that cardiac eicosanoids were significantly reduced by fish oil and this subsequently reduced ventricular fibrillation.

Chartnock and colleagues[45] studied the effect of n-3 PUFAs on the cardiac performance in marmoset monkeys and found the dietary treatment to be beneficial. Both the mechanical performance and the electrical stability of the animal hearts improved by n-3 PUFA diet. Replacing saturated animal fat in the diet with either n-6-rich sunflower seed oil or n-3-rich fish oil reduced the incidence and severity of arrhythmias occurring during ischemia in rats.[46] Later studies confirmed that dietary n-3 PUFA may reduce the vulnerability of normal or ischemic myocardium to arrhythmias in marmoset monkeys.[47] Diets were obtained by blending sheep fat with sunflower seed (SF/SSO) or fish oil (SF/FO) and a base diet. After 16 weeks feeding, the VF threshold (VFT) was significantly elevated in the SF/FO group compared with the SF/SSO group. Similar observations were made by Pepe and McLennan[48] in rats fed fish oil for 16 weeks. Reperfusion arrhythmias arise when total or partial blood flow is restored to a previously ischemic region of the heart [49]

Rats fed fish oil (menhaden oil) had a significant reduction in ischemic damage and incidence of fatal arrhythmias. This effect may be due to related changes in the fatty acid composition of myocardium phospholipids. Our results in the pig model do not support a major direct effect on myocardial conduction from n-3 PUFA. Although there is considerable release of nonesterified n-3 fatty acids during myocardial ischemia, conduction velocity (predominantly mediated by sodium channels) decreased by similar amounts in the beef– and fish oil–supplemented pigs (unpublished results). To delineate the biochemical basis by which n-3 PUFAs may prevent arrhythmias, it was necessary to turn to cultured myocytes. We have developed a methodology to culture cardiomyocytes from adult porcine heart and have examined incorporation of n-3 PUFAs into phospholipids.[16]

C. *In Vivo* and *In Vitro* Studies

Beneficial effects of n-3 PUFAs in prevention of arrhythmias may be due to their influence on electromechanical and biochemical characteristics of the ventricular myocytes.[50–52] Dietary n-3 PUFAs affect the production of prostaglandin by the heart[53,54] as well as in cultured rat neonatal myocytes.[15] Delerive and co-workers[55] showed that use of culture media modified with DHA increased the DHA content in phospholipids (20%). This modification favored the decrease of cAMP production capacity after hypoxia-reoxygenation, but did not affect the inositide phosphate (IP) production capacity. Thus, DHA appears to reduce the β-adrenergic biochemical response in normoxia and to trigger this decrease in hypoxia, suggesting a "β-blocking-like" effect as one of their mechanism of action. Eicosapentaenoic acid has been shown to have antiarrhythmic effect on induced tachycardia and fibrillation in intact animals and in cultures of isolated myocardial cells.[56] Some studies have shown that docosahexaenoic acid but not eicosapentaenoic acid inhibit ischemia-induced cardiac arrhythmias.[57] The antiarrhythmic potential of EPA or DHA from *in vitro* studies needs to be extended to trials where clinical end points are determined.

V. MECHANISMS OF ACTION OF N-3 PUFAS

The mechanisms by which these PUFAs exert their beneficial effect on cardiac arrhythmias have been intensively investigated in healthy animals, animal models, and *in vivo* and *ex vivo* studies in humans. These mechanisms can be broadly classified as follows:

- Modification of the fatty acid composition of membrane phospholipids
- Effect on eicosanoid metabolism
- Direct effect of nonesterified fatty acids (NEFA) on the myocardium
- Effect on the inositol lipid cycle and cell signaling

All these mechanisms appear to be interrelated and it is possible that the antiarrhythmic role of n-3 PUFAs is a result of interaction of two or more of these mechanisms, but the sequence of action has not been elucidated.

A. Modification of Membrane Phospholipids by n-3 PUFAs

It is now well known that n-3 fatty acids are incorporated into biological membranes after dietary supplementation.[25,58] Modification of the type of fatty acids contained in the phospholipid bilayer may alter fluidity, receptor binding, and eicosanoid generation.[56,60,61] The manipulation of the fatty acid composition of the media has been shown to result in modification of the phospholipid fatty acid profile of the cardiomyocytes.[62–64] Hallaq and colleagues[65] observed that alterations in the fatty acid composition of the culture medium by n-3 fatty acids reduces calcium influx rate by 30% and prevents the toxicity of high concentrations of the cardiac glycoside ouabain to isolated neonatal rat cardiac myocytes. Changes in cardiac phospholipids and the NEFA composition as a result of PUFA

supplementation may have antiarrhythmic effects.[46,66] The ratio of arachidonic acid to docosahexaenoic acid in cardiac membrane phospholipids appears to be a key factor in ischemic damage during cardiac arrhythmias. This AA/DHA ratio in cardiac membranes is frequently much higher than expected as reported by Gudbjarnason and Halligrimsson.[41] The fish oil n-3 PUFA, EPA and DHA, are taken up by the myocardium and incorporated into phospholipids like phosphatidylcholine and phosphatidylethanolamine. This uptake is largely at the expense of arachidonic acid.[67] Receptors involved in cellular signaling, transporters, and enzymes are embedded in the membrane lipid bilayer and any changes to the fatty acid composition of the membrane may affect their functions.

B. Effect of n-3 PUFAs on Eicosanoid Metabolism

In addition to incorporation into cell membrane structural components, n-3 and n-6 polyunsaturated fatty acids are precursors of cellular signal molecules such as prostaglandins, thromboxanes, leukotrienes, lipoxins, epoxides, hydroxyeicosatetraenoic acids (5-HETE and 12-HETE), and diol forms of HETE (diHETEs). These are hormone-like substances and are usually designated as eicosanoids. They are formed locally and have a very rapid turnover. Different types of fatty acids give rise to different types of eicosanoids. Eicosanoids are produced in small volumes in normal tissues, but a small change in their metabolism may have an influence on several conditions, for example, atherosclerosis,[25] thrombosis,[68] inflammatory diseases,[69] and possibly in the development of cancer as well.[70] The most common eicosanoids having EPA or AA as precursors are shown in Figure 20.2. The unesterified arachidonic acid is liberated from phospholipids by the action of phospholipase A_2, which may be the rate-limiting step in this cascade. Eicosanoids are local signal molecules, which are important in inflammatory, allergic, and immune responses and platelet aggregation.[37,71–73]

Eicosanoids derived from n-3 fatty acids generally differ in their biological functions from those derived from n-6 fatty acids.[74] EPA-derived eicosanoids, such as thromboxane A_3 (TXA_3), prostacyclin I_3 (PGI_3), prostaglandin E_3 (PGE_3), and leukotriene I_5 (LTB_5), reduce blood platelet aggregation, decrease plasma triglycerides and free fatty acids, in addition to many other biological functions. When fish oils are included in the diet, EPA and DHA compete with AA in several ways:

1. They inhibit Δ-6 desaturase activity to inhibit AA biosynthesis.[75]
2. They compete with AA for the sn-2 position in membrane phospholipid thereby reducing plasma and cellular levels of AA.[76]
3. These factors contribute to antiarrhythmic effects of these fatty acids.

C. Effect of Non-Esterified Fatty Acids (NEFA) on the Myocardium

Earlier studies proposed that arrhythmias were metabolically induced by acute lipid mobilization from adipose tissue and results in high free fatty acid (FFA) levels in the plasma and myocardial cells.[77] Under normal conditions, tissue level of NEFA is very low. The majority of fatty acids are oxidized in the mitochondria to provide energy and a small part is esterified and stored in the triacylglycerol and phospholipid pool.[78] After meals, glucose is the preferred fuel for myocardial oxidative metabolism but during fasting NEFA becomes the preferred fuel. During ischemia fatty acid oxidation is disturbed and nonoxidized NEFA accumulates. This accumulation of NEFA is believed to be toxic to the heart and may induce arrhythmias.[79] Elevated levels of myocardial NEFA have also been observed in experimental animals subjected to ischemia.[80]

Recently, Abeywardena and Charnock[81] examined the effects of various fatty acids on myocardial eicosanoids and myocardial phospholipids. Fish oil–fed animals had the lowest proportion of LA and AA with a rise in the n-3 fatty acids in the cardiac membrane, which highlighted the importance of the balance of eicosanoids in determining the arrhythmic outcome in ischemia as well as after reperfusion. A study by Pepe and McLennan[48] showed also that dietary fish oil prevented the initiation and reduced the severity of arrhythmias in the isolated hearts in response

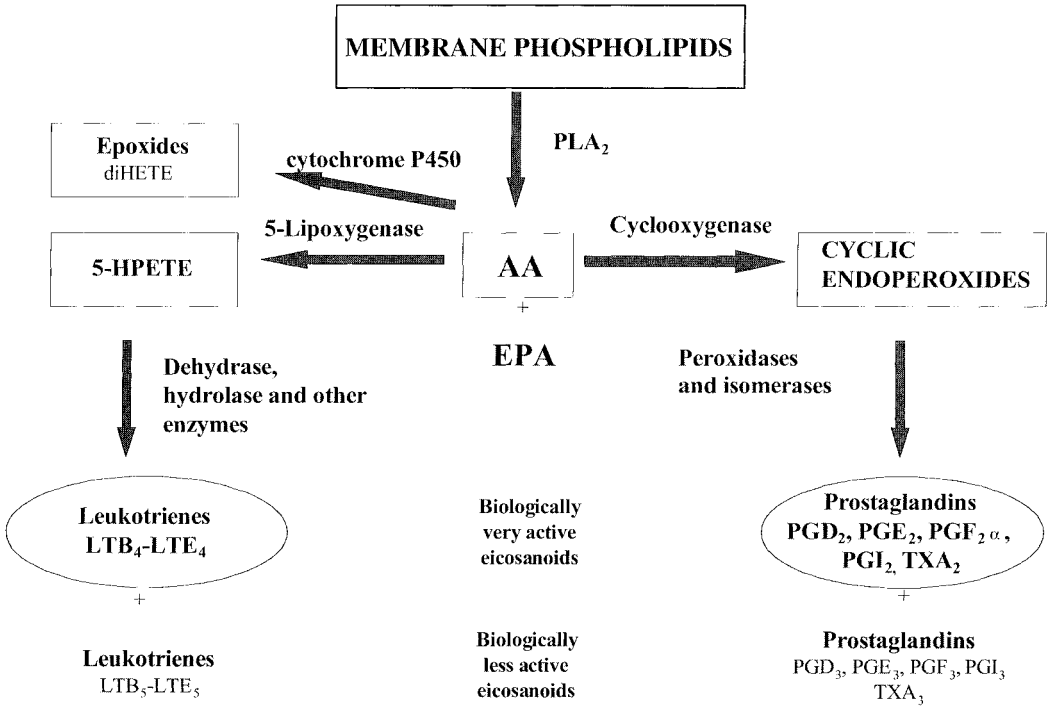

FIGURE 20.2 An overview of some central eicosanoids formed from EPA (shaded circles) and AA (open circles). Less active eicosanoids are presented in normal script (as LTB_5 and TXA_3), and active metabolites (as LTB_5 and TXA_2) are shown in bold script. PGE_2 and $PGE_{2\alpha}$ are mainly produced in kidney and spleen. PGI_2 are dominant in vascular endothelium, kidney, and heart. TXA_2 are produced by platelets and polymorphonuclear (PMN) leukocytes. HPETE are the major lipoxygenase products in basophils, PMN leukocytes, mast cells, and macrophages. LTB_4 are generally involved in regulating neutrophil and eosinophil functions.

to a variety of stimuli. It was speculated that the most likely mechanism for this preventive action was the altered fatty acid composition of myocardial membranes or intracellular NEFA pool. The altered proportions of AA, EPA, or DHA in the myocardial NEFA may lead to alterations in the production of myocardial TXA_2 and the vulnerability of the heart to develop arrhythmia during the partial ischemia.[45] In this study, it was showed that the NEFA even in very low concentration had a direct effect on the heart by reducing the amount of eicosanoids and was one of the major factors that determined the vulnerability of the heart to develop ventricular fibrillation. In a recent study, Kang and Leaf[58] demonstrated that PUFA including AA, EPA, and DHA can bind to highly reactive sodium pump and prevent arrhythmias. Saturated and monounsaturated fatty acids did not significantly bind to the Na^+ channels and were not believed to be antiarrhythmic although LA was found to have a mild effect. They concluded that n-3 PUFA did not have to be incorporated into membrane phospholipids as only free acids exhibited antiarrhythmic potential. Further evidence for the antiarrhythmic effect of free fatty acid was provided by Weylandt and colleagues.[82] Cardiac myocytes were cultured in the presence of EPA and DHA and when NEFA were removed from the medium, no antiarrhythmic effect was observed despite the membrane enrichment with EPA and DHA.

D. Effect on the Inositol Lipid Cycle and Cell Signaling

An important aspect of the membrane lipid metabolism in the cells is the inositol lipid cycle. The inositol lipid cycle generates two important second messengers that are involved in cell signaling, namely, inositol phospholipids and diacylglycerol (Figure 20.3). An extracellular

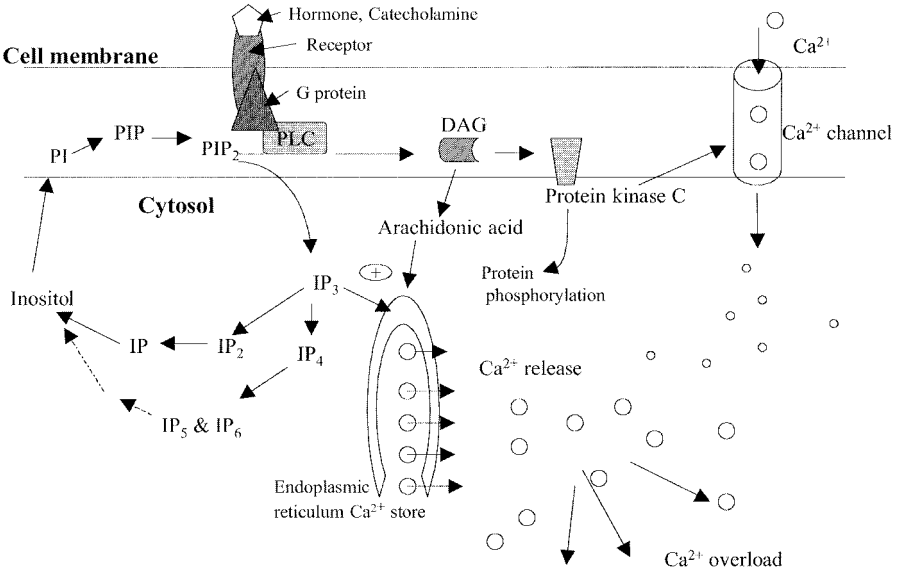

FIGURE 20.3 Signal transduction through the generation of the two second messengers IP_3 and DAG and calcium mobilization.

agonist interacts with a specific cell receptor to form the receptor–agonist complex (RAC) and initiates the inositol lipid cycle. In this cycle, RAC activates a specific phopholipase C that then cleaves phosphatidyl inositols into two important second messengers, IP_3 and DAG. IP_3 activates the release of intracellular calcium ions from the endoplasmic reticulum into the cytoplasm and facilitates entry of Ca^{2+} ions into the cells and may alter electropysiological properties of the cells. In myocytes, the balance of Ca^{2+} ions available for contraction is an important determinant of arrhythmias. Intracellular calcium concentration and disturbances to calcium homeostasis has been associated with arrhythmias.[83]

Alteration in cellular calcium can contribute to the developments in impulse generation and impulse conduction during myocardial ischemia.[5] The major mode of entry of calcium into vascular smooth muscle is through two types of calcium channels, the voltage-operated and the receptor-operated channels.[84] In the voltage-operated channel, intracellular calcium is released in response to the wave of electric activity that spreads rapidly over the heart to initiate each contraction. In the receptor-operated channel, TXA_2, leukotriene B_4 (LTB_4), and cytochrome P450 products of AA amplify an initial Ca^{2+}-related signal for cell activation by stimulating specific membrane receptors coupled to phospholipase C, resulting in increased intracellular Ca^{2+} concentrations.[85] Omega-3 PUFAs have a secondary effect on one or more of the above mechanisms. Kinsella and colleagues[86] demonstrated an increase in Ca^{2+} uptake by endoplasmic reticulum in rats raised on fish oil–enriched diet which was associated with the prevention of arrhythmia. EPA and DHA had been shown to prevent the toxicity of high concentrations of the cardiac glycoside ouabain to isolated neonatal rat cardiac myocytes. Arachidonic acid [C20:4 (n-6)] lacks such protective action. The protective effect of the n-3 fatty acids is associated with their ability to prevent high levels of cytosolic free calcium from occurring in response to the ouabain.[65,87] These authors also believe that the n-3 PUFAs are not only acting as calcium channel blockers but also as modulators or "valves" that control the influx and efflux of calcium to maintain normal contractility of the myocytes.[37] In rat myocytes, n-3 fatty acids reduce calcium fluxes, possibly because their presence in the plasma membrane alters channel structure or function.[35]

In the cardiac myocyte, protein kinases regulate contraction, ion transport, fuel metabolism, and probably gene expression and growth.[88] Protein kinase C (PKC), first identified in 1977, is a

calcium-dependent and phospholipid-dependent serine and/or threonine specific kinase. DAG generated by receptor mediated hydrolysis of membrane phospholipids, particularly phosphatidylinositol 4,5-biphosphate, activates this enzyme by increasing its affinity for calcium ions. DAG was found to stimulate PKC by decreasing the amount of Ca^{2+} required for activation.[89]

PKC is also believed to be activated *in vitro* by AA and other cis-unsaturated fatty acids.[90] In the absence of phosphatidyl serine (PS), AA and oleic acid may activate PKC to varying degrees. Synergistic action of fatty acids and DAG for the activation of PKC has been reported. Studies on the interaction of fatty acid with DAG and Ca^{2+} have revealed conflicting results. In some studies, activation of PKC by fatty acids is independent of Ca^{2+} and in some cases it is Ca^{2+} dependent. DAG sometimes shows no effect on fatty acid activation of PKC, slightly modifies the effect of fatty acids or strongly synergizes with fatty acids.[91] After activation PKC apparently translocates to the membrane in calcium-dependent manner.[92] Once bound to the membrane, PKC is believed to phosphorylate a number of proteins. In the membrane, crucial roles have been assigned to PKC in downregulation of receptors, modulation of ion channels, release of hormones and neurotransmitters, and exocytosis. The role of PUFAs in PKC activation has been studied. McPhail and colleagues[93] tested the ability of a variety of unsaturated or saturated long-chain fatty acids to activate PKC. They found that γ-linolenate and linoleate-activated PKC in the presence or absence of diolein. The potency of the unsaturated fatty acids was proportional to the number of double bonds, in the following order: γ-linolenate (18:3) > linoleate (18:2) > oleate (18:1). In contrast, the saturated fatty acids palmitate (16:0) did not stimulate PKC. Concentrations of stearate (18:0) of as high as 100 μ*M* also failed to activate PKC. A study by Vernhet and colleagues[94] in 3T3 fibroblast cells showed that EPA containing DAG are as potent activators of PKC as AA, and hence does not support the hypothesis of alteration of PKC activity by n-3 PUFAs.

Phosphoinositides constitute 2 to 8% of the membrane lipids. The most abundant form is phosphatidylinositol (PI). The regulation as well as the subcellular distribution of these phosphorylation–dephosphorylation processes is still not clear. In rat ventricular myocytes, epinephrine is most potent agonist at eliciting inositol phosphate release. (1,4,5)IP_3 is now well recognized as the second messenger that is able to release Ca^{2+} from nonmitochondrial pools, thus promoting the Ca^{2+}-induced influx of extra calcium, possibly in conjunction with IP_4. In heart, release of IP_2 appears to predominate over the release of IP_3. It is possible that release of IP_2 serves to substitute for IP_3 release, which is arrhythmogenic in heart muscle.[95] Feeding rats a diet rich in n-3 fatty acids caused a selective reduction in IP_2.[96] Reduction in oxygen tension selectively reduced the content of IP_2 and prevented increase caused by α-adrenoreceptor activation.[97] These two observations indicate a functional role for IP_2 in the heart.

These studies suggest a prominent therapeutic role for fish oil rich in n-3 PUFAs in the prevention and treatment of coronary artery disease. Supplementation with n-3 fatty acids can substantially improve vascular and platelet functions. Although this simple dietary change has the potential to have major public health benefits, it has not been widely adopted. Many of the changes induced by n-3 PUFAs are poorly understood and the public and medical profession remains skeptical about the purported benefits of fish oil supplementation, despite strong epidemiological evidence of benefit. Until the mechanisms of action of these fatty acids are fully understood, this skepticism is likely to remain.

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21 Omega-3 Fish Oils and Insulin Resistance

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I. INTRODUCTION

The question of whether n-3 fatty acids affect glucose homeostasis and insulin action is clinically relevant. Patients who have diabetes or who are insulin resistant may be asked to supplement their diet with either fish or n-3 fish oils for several reasons; i.e., to treat hypertriglyceridemia, to reduce the risk for atherosclerosis, or even to prevent diabetes mellitus.[1,2] However, some studies show that n-3 fish oils may have adverse effects on the glycemic control.[3,4] Therefore, it is important to evaluate carefully our current knowledge about the effects of n-3 fish oils in diabetes mellitus.

Normal glucose homeostasis is achieved by a delicate balance among pancreatic insulin secretion, hepatic glucose production, and peripheral glucose utilization. A change in any one of these can be compensated for by an alteration in another. For example, impaired insulin action in the peripheral tissues can be compensated for by increased pancreatic insulin secretion and hyperinsulinemia. In contrast, improvement in peripheral insulin action may result in decreased pancreatic insulin secretion.

Effects of n-3 fish oils on glucose homeostasis have been studied at various levels. Their overall effect on glycemic control is assessed by measuring blood glucose, glycosylated hemoglobin (Hgb A_1C) levels, and urine glucose excretion. Secretion of insulin from the pancreas is determined by measuring insulin and C-peptide responses to administration of oral or intravenous glucose, mixed meal, and by hyperglycemic insulin clamp technique. Hepatic glucose production and peripheral insulin action are assessed by the euglycemic clamp technique and by simultaneous use of radioisotopes. All these assays and techniques have been adapted to animal models. Furthermore, studies in *in vitro* systems helped to identify the cellular and molecular mechanisms of the biochemical effects of n-3 fish oils.

In this chapter, effects of n-3 fish oils on the overall glycemic control and the individual components of the glucose homeostasis (pancreatic insulin secretion, hepatic glucose production, peripheral glucose utilization) are reviewed.

0-8493-8734-5/01/$0.00+$.50
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II. EFFECTS OF N-3 FISH OILS ON GLYCEMIC CONTROL

Recently, ethyl esters of EPA and DHA have become available for the treatment of hypertriglyceridemia. Since hypertriglyceridemia is the most common lipid abnormality associated with diabetes mellitus and an integral component of the insulin-resistance syndrome, safe and effective use of n-3 ethyl esters has been evaluated. In a large, multicenter study by Sirtori and colleagues,[5] 203 patients with type 2 diabetes were treated with n-3 ethyl esters, while 211 received the placebo oleic acid ethyl ester. There was no change in fasting plasma glucose, HgB A_1C, or insulin levels. This study also included 103 patients with impaired glucose tolerance. Of these subjects, 52 were randomized to receive n-3 ethyl esters. When glucose tolerance tests were repeated after the treatment, there was no change in the total area under oral glucose tolerance curve. Most other studies tested the effects of native fish oil extracts. Toft and colleagues[6] compared the effects of corn oil and n-3 fish oils in 78 individuals with non-diabetic hypertension and found no change in fasting plasma glucose, HgB A_1C, or insulin levels. Sheehan and colleagues[7] investigated the effects of fish oils in 15 patients with non-insulin-dependent diabetes and showed that there was no change in the fasting or postprandial glucose, fasting insulin, or HgB A_1C. Earlier studies in subjects with diabetes provided variable results possibly due to the differences in the initial glycemic control or the dose of n-3 fish oil supplementation.[3,4] Invariably, effects of fish oils were always reversible after the discontinuation of n-3 fish oil.[3,4] We reported that fish oil supplementation transiently increased HgB A_1C in patients with type 2 diabetes.[8] However, this was reversed despite continuation of fish oil treatment. Connor and colleagues[9] compared effects of fish oil supplementation with olive oil placebo in 16 patients with type 2 diabetes and found that fasting glucose, HgB A_1C, 24-h urinary glucose, fasting plasma C-peptide, and 24-h urinary C-peptide concentrations had not changed. However, as reviewed by Malasanos and Stacpoole,[4] n-3 fish oil supplementation had unfavorable effects on both fasting- or meal-stimulated glucose and glycosylated hemoglobin levels in several other earlier studies (Table 21.1).

III. EFFECTS OF N-3 FISH OILS ON PANCREATIC INSULIN SECRETION

The current understanding of insulin secretion is that glucose and other secretagogues act at cell surface receptors and modulate the concentrations of several second messengers, such as cyclic AMP, calcium, and diacylglycerol, which act through protein kinase (PK) A, calcium-calmodulin-dependent PK, and PKC, respectively.[10] Specific insulin secretagogues may use specific second messengers. For example, glucose-mediated insulin secretion probably does not require either PKA or PKC. Thus, insulin response to a glucose load may be different from the response to a mixed meal. Metabolic products of arachidonic acid and various prostaglandins may also play significant roles in insulin secretion.[11,12] In many metabolic pathways, i.e., cyclooxygenase and lipoxygenase, n-3 fish oils compete with arachidonic acid.[13] Thus, it is conceivable that n-3 fish oils decrease insulin secretion from the pancreas by altering metabolism of arachidonic acid. Consistent with this hypothesis, several studies show that n-3 fish oil supplementation is associated with a decrease in insulin secretion. Glauber and colleagues[14] reported that both meal-stimulated and glucagon-stimulated insulin secretion were diminished during fish oil treatment. Stacpoole and colleagues[15] also observed a similar decrease in insulin secretion in response to a glucose tolerance test in healthy subjects. More recently, Delarue and colleagues[16] reported decreased insulin, but not C-peptide, response to oral glucose or fructose challenge. Friday and colleagues[17] did not find a consistent decrease in meal-stimulated insulin secretion in patients with type 2 diabetes.

Annuzzi and colleagues[18] reported a normal insulin secretory response to a mixed meal, intravenous glucose, and arginine in patients with type 2 diabetes. Similarly, Fasching and colleagues[19] found normal insulin secretion in response to intravenous glucose injection in patients with impaired glucose tolerance. The recent study of Toft and colleagues[6] determined the effects

TABLE 21.1
Effects of n-3 Fish Oils on Various Components of Glucose Homeostasis

	Observations	Ref.
	Insulin Secretion	
Humans	Decreased	14–16
	No change	6, 17–19
Animals	Increased	20
	Decreased	21
	No change	22
	Hepatic Glucose Production	
Humans	Increased	14, 23
	No change	19, 24
Mechanisms	Increased glucose production from glycogen	
	Decreased G-6-phosphatase	23
	Decreased G-6-PDH	25
		25
	Insulin Action	
Humans	Increased	19
	No change	14, 18, 24
Animals	Increased	28–30, 32–34
	No change	34
Mechanisms	Increased GLUT 4	28, 30
	Decrased GLUT 4	29
	Increased insulin binding	35
	Increased glucose transport	33, 36, 39, 40
	Increased glucose oxidation	32, 33
	Increased lipogenesis from glucose	32, 33, 38

of corn and n-3 fish oils on insulin secretion both by oral glucose tolerance test and hyperglycemic clamp and found no difference. To the author's knowledge there is no study in humans that shows that n-3 fish oils increase insulin secretion. On the other hand, in animals increased as well as decreased insulin secretory responses have been reported. For example, Miura and colleagues[20] compared the effects of lard to that of fish oil in the genetically diabetic db/db mice. Fish oil decreased fasting blood glucose, as well as the glucose levels after 30 and 60 min during the oral glucose tolerance test. Plasma insulin levels were significantly increased after the 30 min. There was also a more-pronounced hypoglycemic effect of insulin after 60 min. Both dietary treatment groups had similar body weights. Therefore, these results suggested that n-3 fish oils may improve the glucose tolerance both by increasing secretory capacity of the pancreatic beta cells and improving insulin resistance. In contrast, Chicco and colleagues[21] investigated the effects of cod liver oil on insulin secretion in Wistar rats and found a decrease in pancreatic insulin secretion without any changes in the glucose tolerance curve. There was no concomitant decrease in the insulin content of the pancreas. Lombardo and colleagues[22] also studied the effects of cod liver oil on insulin secretion and glucose disappearance rates in sucrose-fed rats. These were long-term feeding studies extending to 120 days. As expected, sucrose feeding caused severe insulin resistance and cod liver oil reversed the resistance by improving the glucose disappearance rate, but there was no change in the insulin response to the intravenous glucose load.

IV. EFFECTS OF N-3 FISH OILS ON HEPATIC GLUCOSE PRODUCTION

The hepatic glucose output is regulated by the rates of (1) glycogen synthesis, (2) glycogen breakdown, (3) gluconeogenesis, and (4) glycolysis. Studies in humans suggest that n-3 fish oils may increase hepatic glucose production.[3] Even when there is no change in the total glucose output, contribution of glycerol to the hepatic gluconeogenesis may be increased. It is postulated that repartitioning of glycerol from triglyceride synthesis to gluconeogenesis and increased oxidation of lipids may be responsible for the increase in hepatic glucose output. Puhakainen and colleagues[23] compared the effects of 12 g of fish oil supplementation with those of corn plus other oils in patients with type 2 diabetes. Gluconeogenesis from ^{14}C-glycerol increased by 32%. However, overall glucose production from ^{3}H-glucose, the glycemic control, and fatty acid oxidation remained unchanged. Glauber and colleagues[14] also reported a 26% increase in hepatic glucose production during fish oil treatment in patients with type 2 diabetes mellitus, whereas Borkman and colleagues[24] and Fasching and colleagues[19] did not observe any increase in hepatic glucose output in patients with type 2 diabetes or impaired glucose tolerance, respectively. Mechanisms of n-3 fish–oil induced changes in hepatic glucose output have been further explored in animal models. Chiang and Tsai[25] compared the effects of fish oils to that of lard in rats and found that fish oil did not change the fasting plasma glucose level. However, fish oil decreased the hepatic glucose 6-phosphatase and glucose-6 phosphate dehydrogenase (G-6-PDH) activities. There was also an increase in liver glycogen concentration. Taken altogether, these findings suggested a possible decrease in the breakdown of glycogen to glucose. Liver total lipids were decreased. Along with the decrease in the G-6 PDH activity, this may suggest a decrease in the synthesis of lipids in the liver.

V. EFFECTS OF N-3 FISH OILS ON PERIPHERAL INSULIN ACTION

As recently reviewed by Combettes-Sourvain and Issad[26] the current understanding of insulin action is that transport of glucose into the cells and several other metabolic actions of insulin start with the binding of insulin to the insulin receptor. This is a plasma membrane glycoprotein with 2 α and 2 β subunits. The α subunits bind insulin, and β subunits have tyrosine kinase activity. After binding of insulin to the receptor and autophosphorylation of the β subunits on tyrosine residues, the receptor becomes a docking site for the insulin receptor substrates (IRS 1–4). Insulin receptor substrates activate the mitogenic functions of insulin through the mitogen activated protein (MAP) kinase cascade and the metabolic functions through the phospho inositol 3 kinase (PI 3-K) cascade. The PI 3-K activates protein kinase B (PKB), which facilitates the transport of glucose by recruiting glucose transporters (GLUT) to the plasma membrane, the glycolysis by activation of 6-phospho-fructo 2-kinase, and glycogen synthesis.

In humans, Glauber and colleagues,[14] Borkman and colleagues,[24] and Annuzzi and colleagues[18] did not demonstrate any change in the peripheral uptake of glucose in patients with type 2 diabetes. On the other hand, Fasching and colleagues[19] showed improved glucose uptake in subjects with type 2 diabetes and impaired glucose tolerance, respectively. Omega-3 fish oils may affect insulin action at several levels. At the insulin receptor, fatty acids in the plasma phospholipid bilayer determine the physical chemical properties of the membranes and cellular function, including response to the hormones. Field and colleagues[27] showed that diabetic state decreases polyunsaturated fatty acid content of adipocyte plasma membrane phospholipids, particularly arachidonic acid. An increase in the polyunsaturated/saturated fatty acid ratio of the cell membrane increases the membrane fluidity, as well as insulin binding to the receptor and insulin action. However, this effect does not seem to be specific to n-3 fatty acid; n-6 polyunsaturated fatty acid also induced such an improvement. In a model of a spontaneously diabetic rat called Otsuka Long-Evans Tokushima Fatty Rat, fish oil supplementation improved insulin action as measured by euglycemic insulin glucose clamp technique.[28] When fish oils were compared with other fatty acids, such as olive oil, safflower oil, and lard, olive oil and safflower oil had neutral effects on the insulin

resistance, whereas lard exacerbated the insulin resistance. These differences occurred even though all oil-fed groups had similar body weights. The analysis of the skeletal muscle phospholipid composition showed that fish oil–treated rats had higher EPA and DHA concentration, but lower arachidonic acid concentration. Furthermore, there was a significant increase in GLUT 4 mRNA in the skeletal muscle. This was in contrast to the findings of Sebokova and colleagues[29] who reported a decrease in GLUT 4 protein in sucrose-fed rats. Storlien and colleagues[30] showed that in Wistar rats diets high in saturated, monounsaturated, and n-6 polyunsaturated fatty acids all caused severe insulin resistance, as determined by euglycemic clamp. Omega-3 fatty acids from fish oils, but not α-linolenic acid, prevented insulin resistance both in the liver and the periphery. Omega-3 fish oil content of the muscle phopholipids correlated directly with insulin action in that muscle. In this study all experimental groups had similar body weights.

A reason for the improvement of insulin action may be that polyunsaturated fatty acids of n-3 class induce less weight gain. Storage of marine oils in the adipose tissue is less than their amount present in the diet, suggesting that these fatty acids are not stored well in the adipose tissue. This may be due to the fact that n-3 fatty acids may be preferentially oxidized, rather than stored. Hill and colleagues[31] demonstrated that fish oil–fed rats had less total body fat and less intra-abdominal fat than the lard– or corn oil–fed rats. Rizkalla and colleagues[32] showed that fish oil increased the insulin-stimulated glucose oxidation and glucose incorporation into the lipids in adipose sites in the sucrose-fed rat model. However, in this study the n-3 fish oil–fed animals were also leaner and it was not clear whether the beneficial effects of n-3 fish oils were due to the decreased body weight. In a follow-up study from the same group, Luo and colleagues[33] used pair-feeding to avoid the differences in body weight and demonstrated that, when Sprague-Dawley rats were rendered insulin resistant by sucrose feeding, fish oil increased the insulin-stimulated glucose transport and glucose oxidation and incorporation of glucose to total lipids as compared with corn oil in isolated adipocytes. Insulin action correlated with the fatty acid unsaturation index of the membrane phospholipids, and menhaden oil is incorporated into the adipose site membrane more effectively than the corn oil. Fish oil feeding also corrected the hyperinsulinemia caused by sucrose. In this study, even though the animals were pair-fed, fish oil–fed animals had smaller epididymal fat pads. Podolin and colleagues[34] investigated the sequential effects of n-3 fish oil feeding on sucrose-induced insulin resistance in the Wistar rats. Euglycemic and hyperinsulinemic clamp studies were supplemented with the use of radioisotopes, ^{3}H-glucose and ^{14}C-2-deoxyglucose. Although n-3 fish oil prevented the sucrose-induced insulin resistance in rats, it failed to reverse the insulin resistance once it had established. This was not due to the irreversibility of the sucrose-induced insulin resistance because, when these animals resume the starch based diet, insulin resistance disappeared. These studies showed that fish oils may be more effective in preventing diet-induced insulin resistance than reversing it. Huang and colleagues[35] studied the effect of fish oils in the fructose-fed rat model, which is also insulin resistant. In this model, fructose reduced both insulin binding to adipocytes and glucose uptake. Omega-3 fish oil supplementation reversed these changes.

Another commonly used insulin resistance model has been high-fat feeding. Hainault and colleagues[36] found that 15% fish oil increased the insulin stimulated glucose transport in the adipocytes. However, Storlien[7] reported that in high-fat-fed animals, replacement of 6% of the fatty acids with fish oils increased the *in vivo* insulin-stimulated glucose metabolism in the liver and skeletal muscle, but not in the adipose tissue. Vrana and colleagues[38] used the models of both high-fat and high-sucrose feedings and demonstrated that partial replacement of olive oil with fish oil increased the insulin-stimulated glucose incorporation into adipose tissue lipids. However, this improvement may be transient as reported by Ezaki and colleagues,[39] who showed that although n-3 fish oils initially improved the glucose transport and increased GLUT 1 and GLUT 4 protein, they could not prevent the enlargement of adipocytes and the eventual decline of glucose transport and GLUT proteins.

Insulin resistance can be created also by infusion of tumor necrosis factor (TNF). In this model Sierra and colleagues[40] measured insulin action by hyperinsulinenemic euglycemic clamp

technique, supplemented with ^{3}H-glucose and ^{14}C-deoxyglucose. While there was no decrease in ^{14}C-deoxyglucose uptake in the n-3 fish oil group, there was a significant decrease in the safflower-fed group. These results suggested that fish oil significantly improved the peripheral glucose uptake.

Another mechanism by which fish oils may improve insulin action may be the correction of hyperinsulinemia. Del Prato and colleagues[41] showed that chronic slight hyperinsulinemia created by exogenous insulin infusion or by stimulation of endogenous insulin secretion led to the development of insulin resistance in humans. Correction of hyperinsulinemia by fish oil feeding has been a consistent finding in sucrose-fed animals, as well as high-fat-fed rats.

VI. CONCLUSION

There has been an increasing interest in the effects of n-3 fish oils because of the recent commercial availability of ethyl ester preparations for therapeutic purposes. In general, the available data suggest that n-3 fish oils do not significantly worsen glycemic control. They may either decrease or not affect pancreatic insulin secretion. They may also improve insulin action in the peripheral tissues. Although there are no data to discourage their use in patients with diabetes, there is no evidence to support that use of n-3 fish oils improve the diabetic state either.

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22 Omega-3 Polyunsaturated Fatty Acids and Rheumatoid Arthritis

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I. INTRODUCTION

Rheumatoid arthritis (RA) is a disease that is primarily treated in an ambulatory care setting. The clinical course of RA is characterized by highly variable periods of remission and relapse with some patients stabilized while others develop aggressive disease.[1] Subjects with RA require continued drug treatment to alleviate symptoms and to delay disease progression. Most of the drugs used are expensive and have significant side effects, which involve damage to the liver, kidneys, gastrointestinal tract, and eyes.[2] The nutritional implications are fatigue, lethargy, depression, and hand handicap, as well as altered appetite, nausea, vomiting, taste changes, and altered nutrient metabolism.[3-6] These nutritional changes add to the detrimental effects of accepted RA pharmacologically based therapies. Diseases such as occlusive vascular disease and diabetes mellitus utilize a therapeutic dichotomy of drug therapy and dietary manipulation. This combination of therapies is not a common practice in RA treatment modalities.

0-8493-8734-5/01/$0.00+$.50
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Rheumatologists have been unwilling to embrace dietary manipulation because of the abundance of fads and myths concerning dietary therapy and the willingness of patients with RA to experiment with any regime that promises to improve quality of life. This willingness to experiment is reflected in the results of a U.K. survey in which 75% of respondents believed that food played a significant role in disease progression.[7] In a survey in Australia, 60% of respondents reported that they had used a dietary regimen in an endeavor to ameliorate their RA symptoms.[8]

Dietary manipulation in RA can be either an elimination or supplementation regimen. In the elimination approach, foods associated with amelioration of symptoms such as oils, fish, and fresh vegetables are increased in the diet and those associated with a deterioration in symptoms such as citrus fruit, chocolate, alcohol, red meat, flour products, spices, and carbonated drinks are eliminated.[3,9,10] Elimination studies have not yet been able to provide significant evidence of improvement.[11] A supplementation approach involving the use of long-chain n-3 fatty acids derived from fish oil has provided the most promising results to date.[11,12] Supplementation with n-3 fatty acids has been reported to reduce joint stiffness and pain as well as to reduce the severity and the risk of development of RA.[14-20]

The mechanism involved in fish oil supplementation is based on the fact that dietary n-6 and n-3 fatty acids are the primary modulators of the lipid composition of membrane phospholipids. Fatty acids in the membrane phospholipids are the precursors of eicosanoids, which are important mediators of inflammation, cytokine synthesis, and cell communication.[21] The modern Western diet contains an excess of n-6 fatty acids and a low level of n-3 fatty acids.[22,23] Supplementation of n-3 fatty acids in conjunction with a high dietary intake of n-6 fatty acids does not appear to be effective in increasing cellular levels of n-3 fatty acids.[24,25] The key to supplementation is to limit the intake of n-6 fatty acids in the diet so that the n-6 to n-3 balance approaches 1.[26] There is considerable variation in activity of the eicosanoids derived from n-6 and n-3 fatty acids. The n-6-derived eicosanoids exhibit proinflammatory activities, potent chemotactic activity, vasodilation, and increased vascular permeability.[21] The n-3-derived eicosanoids are anti-inflammatory and much less active as well as poorly synthesized. Thus, n-3 fatty acid supplementation can alter the balance of n-6 to n-3 eicosanoids to produce decreased inflammatory activity.[26]

In the past, pharmacological doses (1.0 to 7.1 g/day) of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) derived from 16 to 20 g/day of fat, either as a triglyceride or an ethyl ester, have been used in clinical trials with few recommendations concerning the quality of fat in the background diet. This is an important consideration because the n-6 fatty acid arachidonic acid (AA) is an EPA antagonist. A recent study in our group was undertaken to determine the potential for dietary manipulation and supplementation in the treatment of RA. A modest improvement ($P < 0.02$) was achieved through the reduced level of n-6 fatty acids in the background diet (<10 g/day) in combination with n-3 fatty acid derived from fish oil supplementation at a dose rate of 40 mg/kg body weight/day, however, these results were more significant than results from previous studies despite the high loss of subjects to the study through medication change and noncompliance. The potential benefits of dietary manipulation and supplementation include the amelioration of RA symptoms and a reduction in health care costs. The most recent cost estimates of RA care (in the United States) for 6.5 million American RA sufferers is $14 billion/year.[27]

II. RHEUMATOID ARTHRITIS

A. Classification of RA

RA is an autoimmune disease, characterized by a chronic inflammatory synovitis of unknown origin. The distinctive features of RA are chronic, symmetrical, and erosive arthritis of the peripheral joints. Previously, RA was regarded as a benign, nonfatal disease; however, it is now accepted that RA reduces life expectancy by 3 to 10 years in both men and women.[28] Twice as many women as

men are affected.[28] The peak onset of RA is in the fourth and fifth decade of life.[28] Mortality rates are higher in those patients who have more persistent joint inflammation, seropositivity for rheumatoid factor (RF), functional loss, and lower levels of education.[29] Patients with RA experience a range of lifestyle diseases, as do the general population; however, they die at a younger age.[30] The primary risk factors for reduced longevity are the greater number of involved joints, cardiovascular comorbidities, older age, lower educational level, and poor functional status.[31] Care of patients with RA requires regular monitoring of disease progression and the effects of treatment. Both regular monitoring and the cost of pharmacological therapies as well as the deleterious effect of these drugs make expenditure on this group a major component of health care costs.[27] New treatment modalities are proving to be very expensive to develop and use.[32]

It is difficult to define RA because there is no single clinical, laboratory, or radiological marker that is specific for the disease. McCarty[33] maintains that the difficulty posed by the need to classify RA results from the wide range of modes in which RA presents as inactive, mild, and self-limiting or quite severe. Additionally, there is a need to distinguish RA from a range of other destructive arthropathies. The current classification scheme is the 1987 American College of Rheumatology (ACR) criteria (Table 22.1).[34] These criteria were developed from data collected from outpatient clinics and assist in the classification of RA as distinct from other inflammatory arthrides.[34]

B. Epidemiology of RA

Normally, disease occurrence can be determined through the measurement of incidence of RA (the rate of new cases arising in a given period) and prevalence (the number of existing cases). Both methods of measurement present difficulties, because of the low overall incidence of RA necessitating large sample sizes with prolonged follow-up times to ensure statistical precision. The incidence rate trend suggests that the incidence and severity of RA is declining.[35] The annual incidence

TABLE 22.1
The 1987 Classification Criteria and Definitions for RA[a]

Criteria	Definition
1. Morning stiffness	Morning stiffness in and around the joints, lasting at least 1 h before maximal improvement
2. Arthritis of 3 or more joint areas	At least three joint areas simultaneously have had soft tissue swelling or fluid (not bony overgrowth alone) observed by a physician; the 14 possible joint areas are right or left PIP, MCP, wrist, elbow, knee, ankle, and MTP joints
3. Arthritis of hand joints	At least one area swollen (as defined above) in a wrist, MCP, or PIP joint
4. Symmetric swelling (arthritis)	Simultaneous involvement of the same joint areas (as defined in 2) on both sides of the body (bilateral involvement of PIPs, MCPs, or MTPs is acceptable without absolute symmetry)
5. Rheumatoid nodules	Subcutaneous nodules, over bony prominences, or extensor surfaces, or in juxta-articular regions, observed by a physician
6. Serum rheumatoid factor	Demonstration of abnormal amounts of serum rheumatoid factor by any method for which the result has been positive in <5% of normal control subjects
7. Radiographic changes of rheumatoid	Radiographic changes typical of rheumatoid arthritis arthritis on posteroanterior hand and wrist radiographs which must include erosions or unequivocal bony decalcification localized in or most marked adjacent to the involved joints (osteoarthritis changes alone do not qualify)

[a] For classification purposes, a patient shall be said to have rheumatoid arthritis if he or she has satisfied at least four of these seven criteria. Criteria 1 to 4 must be present for at least 6 weeks (Arnett et al., 1988).

PIP = proximal interphalangeal; MCP = metacarpophalangeal; MTP = metatarsophangeal

rate for RA cases diagnosed between 1950 and 1974 has been given as 0.5/1000 for females and 0.2/1000 for males with an increasing incidence continuing into the seventh decade of life.[36] The use of the 1987 ARC criteria to classify RA has resulted in a decrease in the annual incidence rate for females to 0.36/1000 and for males to 0.14/1000. The incidence for males increased with age whereas females plateaued between ages of 45 and 70.[36] Symmons and co-workers[37] claim that the prevalence in Caucasian, European, and North American populations ranges from 0.5 to 2.0% for those over 15 years of age, with age-specific prevalence rates increasing as patients with RA age. The fact remains that, the incidence and severity of RA may be declining over time; evidence suggests that the nature of this change is complex. Seropositive, erosive, and nodular disease in individuals with RA peaked in the 1960s, and has declined since.[38,39]

There are a number of geographic variations observed worldwide. The prevalence estimates for Europe, North America, Asia, and South Africa are quite similar at 0.5 to 1%. Some North American Indians have a disease prevalence of 5%, whereas other native North American Indian populations have a very low prevalence (<0.4%).[40] Solomon and co-workers[41] maintained that the prevalence in urban black African populations suggests a rate similar to Europeans, while rural African populations show a very low prevalence.[41] Low prevalence rates have been reported from Chinese and Indonesian populations.[42,43] Shichikawa's research group[44] reported a low prevalence of RA of 0.4% in Japan. Roberts-Thomson's group[45] found no evidence to suggest that RA in Australian aborigines occurred before and during the early stages of white settlement. Comparisons of prevalence then become problematic if there is a change in the incidence and severity of RA; furthermore, it becomes difficult to determine whether the global variations in prevalence are due to environmental or genetic factors.

C. Genetic Influences in RA

Family genetic studies provide compelling evidence that there is a genetic influence in RA. Aho and his group[46] maintain that twin studies can be prone to biased assessment. However, when Silman and co-workers[47] examined disease concordance rates between monozygotic twins of 12 to 14% and dizygotic twins of 4%, and compared them with the background disease prevalence of approximately 1% for nonrelatives, genetic factors appear to contribute to disease risk.

This twin and familial clustering provided some evidence for a genetic origin of RA. Nepom and Nepom[48] suggested that multiple genes are involved with the histocompatability locus antigen (HLA) region on the chromosome. On chromosome 6, the class II major histocompatability complex (MHC) has an HLA-DR region that is located in the HLA DRB1 locus. DR4 is the serological marker associated with HLA DRB1 locus. The genetic susceptibility of Caucasians in North America and Europe has been identified by the presence of four DR4-positive alleles, Dw4, DR 1.1, DRw15, and Dw14.2. Gao and co-workers[49] suggest that individuals who exhibit HLA-DRB/DR4 have a fivefold relative risk of developing RA compared with individuals in the general population. According to Weyard's group,[50] not all individuals exhibiting this genetic susceptibility develop RA. The Japanese have a relatively high prevalence of HLA-DRw15 (which infers susceptibility) but, paradoxically, display a low prevalence of RA.[44]

D. Environmental Factors in RA

Spector's group[51] claim that the strongest candidates for environmental triggers of RA are the sex hormones. This is suggested by the marked female excess in both the incidence and prevalence of RA. It has been noted that the symptoms of RA often abate during pregnancy, with a subsequent flare in the postpartum period. The factors responsible for remission include estrogen levels and increased levels of pregnancy-associated glycoprotein. Spector's group[52] also maintain that RA occurs less frequently in women taking the oral contraceptive pill (OCP), suggesting that the OCP may have a protective effect. Studies on the effect of hormone replacement therapy (HRT) do not

provide a clear association with risk.[53,54] In males, low testosterone levels are implicated with RA; however, it is unknown whether the low testosterone levels occurred as a result of the disease effect or before the initiation of RA.[55]

Researchers have postulated that RA is induced in genetically predisposed individuals by many different arthrogenic agents such as bacteria and viruses. The implicated viruses include retroviruses, Epstein-Barr virus, rubella virus, and parvovirus. Candidate bacteria are mycoplasma, mycobacteria, and some enteric organisms.[56–59] Additionally, a number of studies have suggested a relationship between socioeconomic status, occupation, urban, industrialized environments and RA. Data gathered from the Norfolk Arthritis Register suggests that there is no association between risk of RA and indicators of socioeconomic status.[37] A more-generalized association between RA and urban, industrialised environments has been proposed but results appear to be in conflict.[60] Data on lifestyle factors also provide conflicting information; however, increased risk of RA has been associated with cigarette smoking according to studies conducted by Silman and co-workers.[61] Recent analysis by Pincus and his group[62] contradicts Symmons and colleagues[37] by claiming that social conditions and self-management are the more powerful determinants of health.

This summarizes the current knowledge of the classification and epidemiology of RA worldwide. RA is rare in Indonesia, rural Africa, and among the Chinese. Genetic differences may explain some of the variation in prevalence; however, environmental factors appear to play a role. Incidence of RA has been studied mainly in Western populations. There is some evidence that the incidence in Western populations may have fallen in young women during the 1960s and 1970s. The peak age of incidence is now in the group older than 50 (postmenopausal), whereas previously peak age of incidence was younger (perimenopausal). Although understanding of the genetic and nongenetic factors that make individuals susceptible to RA has increased over recent years, identification of the initiating agents of the disease continue to be elusive.

E. Treatment Paradigms

The biomedical model has been successful in the treatment of infectious diseases; however, Engel[63] suggested that this model had limited merit in the treatment of RA, a chronic noninfectious disease. McCarty[64] described treatment choices as being nonsteroidal anti-inflammatory drugs (NSAID), including aspirin for 2 years, followed by another NSAIDs after 2 years of treatment, then gold after 2.5 years, antimalarials after 4.5 years, and immunosuppressive drugs after 8.5 years. A reassessment of the traditional biomedical model for RA in the early 1980s has caused a change from the paradigm based on the use of NSAIDs, toward a strategy of early use of disease-modifying antirheumatic drugs (DMARDs).[65,66] The paradigm shift was based on the recognition that NSAIDs were more toxic than originally thought, and that NSAIDs do not reduce disability as effectively as DMARDs.[67] Some DMARDs (hydroxychloroquine and sulfasalazine) are less toxic than some NSAIDs, they reduce disability particularly if used in early treatment, and they are more effective analgesics than NSAIDs over time.[66–70] Thus, the paradigm shift entails the early use of DMARDs before joint damage occurs, in conjunction with continual and/or serial use of one or a number of DMARDs, accompanied by careful monitoring and follow-up. DMARDs have a different mode of action from that of NSAIDs. DMARDs action takes from 4 to 12 weeks to influence symptoms; then they are most effective in reducing abnormal levels of erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), and rheumatoid factor (RF). DMARDs can be classified according to toxicity and efficacy. Choice of DMARDs is usually determined by the severity of RA, presence of comorbidities, age, patient expectations, and lifestyle.[71,72]

Corticosteroid use in the treatment of RA is generally avoided. The use of this class of drugs is controversial because of the long-term side effects that develop after prolonged use. Intravenous pulses of corticosteroids and intra-articular injections can improve the quality of life for some patients with RA. Corticosteroids are used in life-threatening complications of pericarditis and vasculitis. Kirwan[73] recommends that they can be used to reduce the rate of progression of early

disease when used in conjunction with other treatments. Surgical treatment for RA is usually indicated when loss of function is a major problem. Tendon rupture repair and joint replacement relieve pain and restore function.[74]

Dietary supplementation using fish oil–based supplements has emerged as the most-promising area of dietary manipulation in RA. Kremer and Bigaouette[5] reviewed a number of studies and reported that fish oil supplementation reduced joint pain. The mechanism of amelioration involved the inhibition of proinflammatory eicosanoids and cytokines.[75,76] Shapiro's group,[20] in a well-designed population-based case-control study in women, found that two or more servings of baked/broiled fish per week were protective in RA prevention. A population study of a fish-eating society in the Fareo Islands reported the prevalence of RA was 1.1%.[19] The high functional capacity and lower occurrence of rheumatoid nodules and erosions compared with previous studies in northern European societies suggest that RA takes a milder course in the Fareo Islands population. An advantage of dietary manipulation is that the inclusion of two or more fish meals per week is an inexpensive modifier of eicosanoid production.

Complete remission from RA is rare. RA generally necessitates the continued use of DMARDs and associated monitoring.[77] Current treatments in trial have developed through the greater understanding of the pathogenesis of RA. Some of the immunological strategies that have been developed are monoclonal antibodies to tumor necrosis factor-alpha (TNF-α), receptor antagonists, and anti-inflammatory cytokines, which may permit a more successful approach to the treatment of RA to be developed; however, these are very expensive.[78]

Proactive attitudes to research are important in view of current health policy, which is concerned with cost containment through limiting patient access to various services. Generally, rheumatological diseases are treated in ambulatory care settings; however, the high prevalence of the disease, the aging of patients, and the long duration of costly management make cost an important consideration in the treatment of RA.[27,32,77]

III. LIPIDS IN THE WESTERN DIET

Lipids are important components of the diet and are a source of energy. They are composed of fatty acids of different chain lengths with different degrees of saturation, as well as varying configurations of the double bonds. The degree of unsaturation in lipids is of great interest because of the health implications of consumption of saturated fats. Dietary lipids also form an integral part of cell membranes and are metabolized by a series of desaturase and elongase enzymes to form 20-carbon fatty acids, the precursors of eicosanoids.[23]

A. Fatty Acids

Triacylglycerides are the most important storage form of fats. Structural lipids and phospholipids have a fairly conservative fatty acid composition with a high proportion of unsaturated fatty acids; however, storage lipid composition is more variable, reflecting the composition of the diet in simple-stomached animals such as humans.[79] An important aspect of lipids in the living organism is their dynamic state. In all parts of the organism, lipids are continually broken down or removed from tissue and replaced. This continual breakdown and resynthesis is called turnover. Turnover of lipids allows a finer degree of control than would be possible in a more static system.[79] The formation of eicosanoids is an example of tightly controlled enzymic peroxidation of lipids. Peroxidation reactions can also occur chemically and, unless controlled by the presence of natural antioxidant systems, can give rise to massive cell damage, disintergration, and disease. The physiological effects of eicosanoids are so potent in minute quantities that they need to be generated quickly and destroyed immediately after they have produced their effect by enzymes that convert them into inactive metabolites. Membrane phospholipids can be considered as a storage of essential fatty acids that are immediately available for eicosanoid biosynthesis when required.[80] As the storage of essential

fatty acids is depleted, they must be replaced by new polyunsaturated fatty acids. This in part explains the dynamic turnover of fatty acids in biological membranes, even though eicosanoid formation represents a very small proportion of fatty acid turnover. Several aspects of eicosanoid metabolism and function still need elucidation. These include (1) the mechanism by which the relative proportions of the different eicosanoids are regulated, (2) the significance of changes of dietary fatty acid composition of the whole body eicosanoid production, (3) the quantitative significance of the different pathways and sites of synthesis, and (4) the quantitative relationship between the requirements for essential fatty acids, which are measured in grams, and the daily production of eicosanoids, which are measured in micrograms.[80] Lipids are involved in immunity in a number of ways, principally through their roles in membrane integrity and in providing precursors for eicosanoid formation. Thus, the proliferation of lymphocytes involves rapid synthesis of new membranes; the interaction of antigens with receptors on the lymphocyte surface is influenced by the physicochemical properties of the membrane; and eicosanoids play a key role in inflammatory reactions. Dietary lipids appear to modulate immune reactions, either by modifying the composition of cell membranes and membrane surface components or by influencing the nature of the precursor pool for eicosanoids.[13] Rheumatoid arthritis is an autoimmune disease that appears to by modulated be the fatty acid content of the diet.[13]

B. Unsaturated Fatty Acids

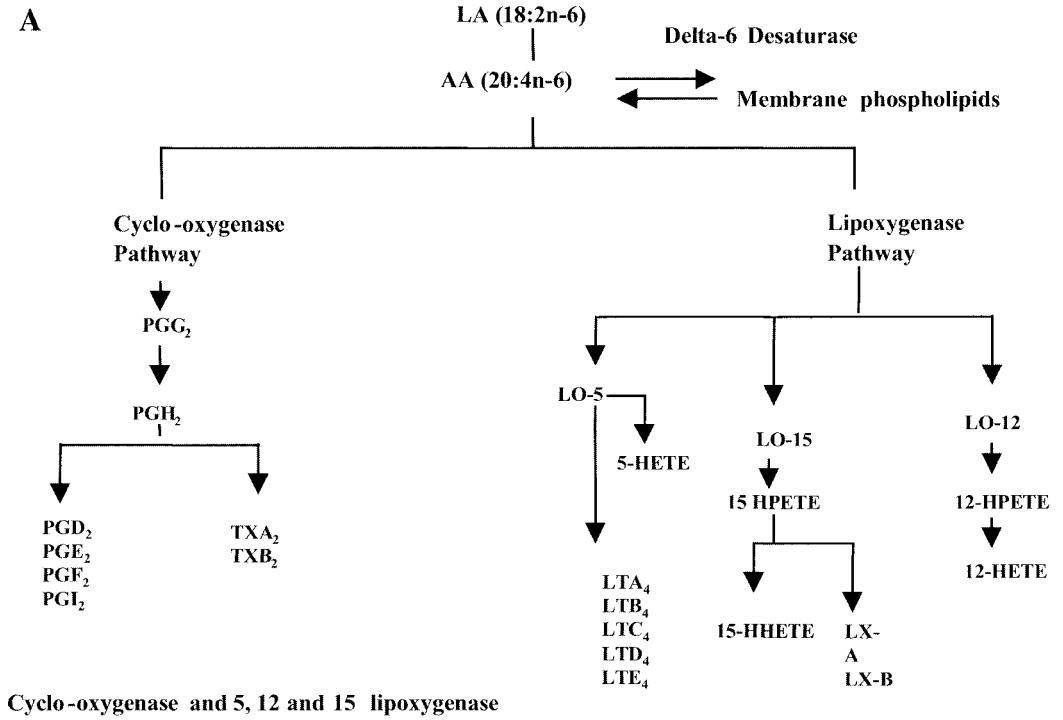
There are three families of fatty acids: saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA), with a subgroup of PUFAs, n-6 and n-3 fatty acids or omega-6 and omega-3 fatty acids. The predominant SFAs in the Western diet are myristic, palmitic, and stearic acids, which are derived mainly from animal fats, dairy products and manufactured foods. The primary MUFA in the Western diet is oleic acid, provided by oils and spreads containing canola, olive, and sunola oils. Mammals are able to synthesize SFA and MUFA *de novo* from simple precursors such as glucose and amino acids. Linoleic acid (LA; 18:2n-6) and α-linolenic acid (LNA; 18:3n-3), the precursors of (n-6) and (n-3), respectively, are essential fatty acids, which cannot be synthesized in the body and, thus, are necessary in the diet. The major PUFA of the Western diet is LA, the food sources of which are vegetable oils such as safflower, sunflower, corn, cottonseed, peanut, and blended oils.[23]

Fatty acids of the n-6 and n-3 subgroup are unique in that they are not interconvertible in mammals. Thus, cellular incorporation of these fatty acids is determined by dietary intake.[79] Fatty acids in the membrane bilayer become substrates for eicosanoids essential for many biological activities including the modulation of inflammation and immune responses (Figure 22.1). Reproducible metabolic alterations in eicosanoid pathways can be manipulated by consistent modification of dietary intake of fatty acids.[80,81]

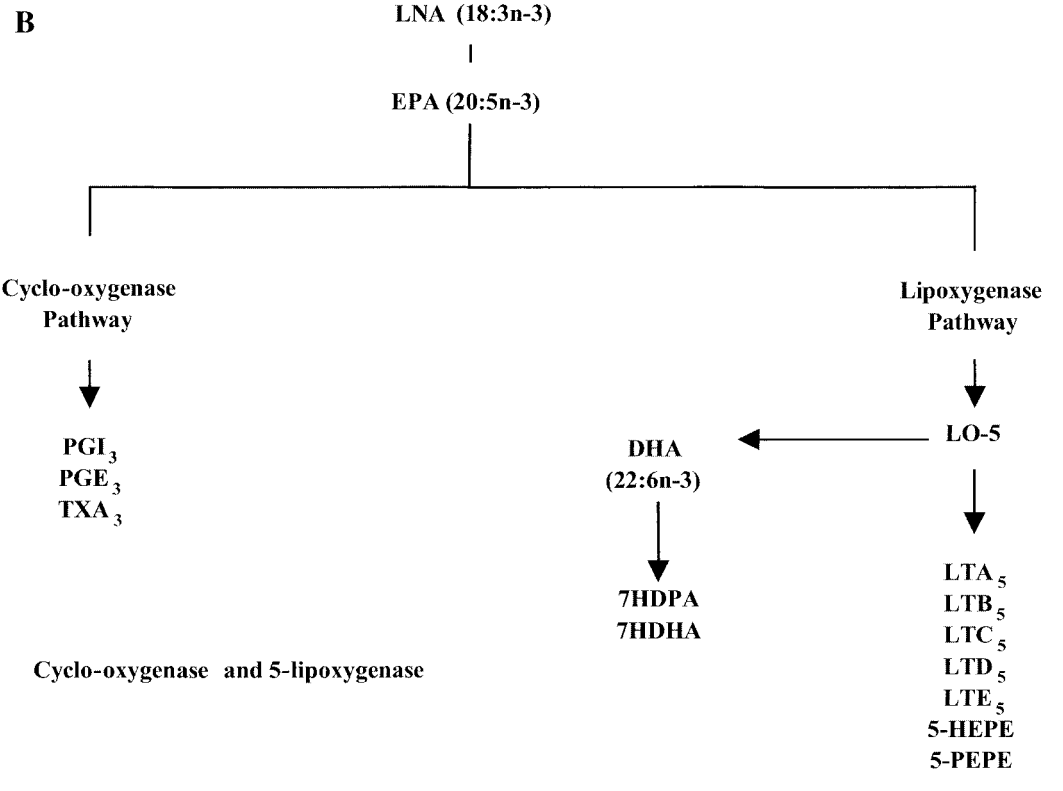
Botanical food sources of LNA are found in green vegetables and vegetable oils such as canola, flaxseed, and soybean. Arachidonic acid (AA; C20:4n-6) is synthesized from dietary LA by desaturation and chain elongation enzymes. Small amounts of AA can be derived from meat, eggs, organ meats, and human milk. Eicosapentaenoic acid (EPA; C20:5n-3) and docosahexaenoic acid (DHA; C22:6n-3) are derived from marine sources such as fish and fish oils. EPA and DHA can also be synthesized by desaturase and chain elongation of LNA. Metabolism of n-3 PUFA (EPA and DHA) and n-6 PUFA (AA) is important because of the action of their eicosanoid metabolites. LA and LNA compete for the same enzyme systems; hence, the dietary balance between n-6 and n-3 PUFA is most important for eicosanoid formation.[82]

C. Dietary Balance of n-6 to n-3 Fatty Acids

Intake of n-6 and n-3 fatty acids in the typical Western diet has been influenced by an overabundance of n-6 fatty acids from seed oils and a suboptimal level of n-3 fatty acids. The balance of n-6 to n-3 in Australia a decade ago was 30 to 1.[83] Where monounsaturated lipid sources are not the



Cyclo-oxygenase and 5, 12 and 15 lipoxygenase
Pathway of linoleic acid metabolism



Cyclo-oxygenase and 5-lipoxygenase

FIGURE 22.1 Metabolic pathways for linoleic acid (A) and linolenic acid (B).

dietary lipid of choice, the balance may still be at the 30 to 1 level. This evidence suggests that it is possible to have 30 times the amount of n-6 fatty acids in the diet, which in turn determines the balance of n-6 to n-3 fatty acids in cell membranes. This shift in n-6 to n-3 balance is important because n-6 and n-3 compete for the same enzyme systems in their conversion to the 20-carbon eicosanoids. These n-6 and n-3 eicosanoids have very different biological activities.[84]

IV. CLINICAL STUDIES AND THE EFFECT OF N-3 FATTY ACIDS ON RA

Experimental studies have provided evidence that the incorporation of n-3 fatty acids into tissues/cells can modify inflammatory and immune reactions. The following discussion traces the development of scientific interest in the anti-inflammatory effects of n-3 fatty acids and the n-3 supplementation studies in RA that have been conducted over the past 16 years.

Studies in animals and humans indicate that changes in dietary intake of essential fatty acids alter cell membrane fatty acids, the eicosanoid profile of stimulated cells, and inflammatory responses.[85] The concept that dietary manipulation may alter symptoms of RA has been treated with distain in the past.[86,87] Most diets have been proposed via anecdotal evidence or personal experience, and acceptance by the general public has depended on the charisma of the marketeer more than scientific evidence. A typical example of this has been the "Dong diet," which was devised by a Dr. Dong as a result of developing arthritis himself.[9] He blamed modern foods and reverted to a traditional Chinese diet which excluded dairy products, alcohol, spices, and additives but included a small amount of meat. Dr. Dong's dramatic recovery provided credibility for his regimen.[88]

A study based on Dr Dong's diet conducted at the University of Florida by Panush and colleagues[89] in 1983 examined the effects of a rice, fish, and vegetable diet on patients. It was a prospective, controlled, blinded study with a restricted diet comparable to a placebo. A small number of patients on the treatment diet did improve significantly from baseline, but the overall improvement in the study cohort was not statistically significant. This study did not involve n-3 fatty acid supplementation, but may have had n-3 fatty acids as a result of the fish-based diet; alternatively, the change from the traditional North American diet high in saturated fat and n-6 fatty acids may have been implicated. The experimental diet was a specific diet free of additives, preservatives, fruit, red meat, herbs, and dairy products.

As interest developed in the concept of dietary manipulation as a treatment modality for RA, investigators initially utilized a crossover design to overcome seasonal variation of the food supply. Kremer's group[15] then reported that a washout period of 4 weeks was insufficient to eliminate the long carryover benefits of dietary fish oil in tissues. This led to the recognition that a noncrossover design was necessary in RA dietary supplementation studies and at least 12 weeks of supplementation to achieve changes in clinical variables of patients with RA.

It became apparent that the investigation protocol was critical in RA supplementation studies. Several investigations of the effect of dietary fish oil supplementation in RA subjects have been conducted in Europe,[90-96] Australia,[18] and North America[14-17,97] using Caucasians of northern European background. Background dietary habits for subjects in these investigations were not reported; however, it was likely that both the fish and n-6 PUFA content of the subjects diets would have been variable considering the geographic and cultural diversity of the regions involved. This factor would have impinged on the significance of clinical change reported in these studies.

Dietary fish oil supplements were compared to a variety of placebo lipids which ranged from inert paraffin wax,[14] corn oil,[17,92] olive oil,[15,16,18,101] and a specially prepared mix of fatty acids designed to replicate the local dietary intake.[91,93,94] It has been suggested that the use of olive oil as a placebo is inappropriate because of its ability to modulate the immune system.[16,18,97] Four groups all reported an improvement in one clinical outcome in the control group.[14,16,92,94,95] Cleland's group[18] reported an improvement in two clinical outcomes in the control group.[18]

Tables 22.2 and 22.3 summarizes 13 studies: 12 of these studies were double blind, placebo controlled; 10 were noncrossover; and the supplemented fish oil was a mixture of EPA and DHA ingested at a daily rate that varied from 1.0 to 7.1 g/day of n-3 fatty acids as indicated in Table 22.1. The outcome measures in most studies included tender joint score, swollen joint score, duration of morning stiffness, grip strength, and patient/physician global assessment of arthritis activity (Table 22.2). Three of the 13 fish oil supplementation studies resulted in an improvement in two clinical measures,[15,90,91] and in three studies at least four clinical measures improved significantly (Table 22.2).[14,16,17] A reduction in tender joint count was the most common favorable outcome in 7 of the 13 studies. Fish oil supplementation significantly decreased NSAID use in the three studies in which it was measured,[91,93,95] and two research groups included complete withdrawal of NSAID use in their study design.[17,92] The results from these studies suggest that there was a modest NSAID sparing effect of fish oil supplementation. Fish oil is not associated with gastric toxicity (as NSAIDs are); thus, it is important from the point of view of reduced toxicity, reduced health cost benefit, as well as efficacy.[98,99]

TABLE 22.2
Comparison of the Effects of n-3 Fatty Acid Supplementation on Clinical Variables

Ref.	Study Design	Duration, weeks	Amount n-3	Clincial Parameters	Effect[a]
Kremer, 1985	Placebo-controlled	12	1.8g EPA	Morning stiffness	+
n = 44/52	Double-blind			Onset fatigue	+
	Randomized			Grip strength	+
	Prospective			Walk time	NE
				Tender joints	++
Kremer, 1987	Placebo-controlled	2 × 14	2.7 g EPA	Morning stiffness	NE
n = 33/40	Double-blind		1.8 g DHA	Onset fatigue	++
	Randomized			Grip strength	NE
	Crossover			Walk time	NE
	Prospective			Tender joints	++
Sperling, 1987	Blind	6	3.6 g EPA	Joint pain index	+
n = 12/14	Prospective		2.0 g DHA	Patient assessment	+
Cleland, 1988	Placebo-controlled	12	3.2 g EPA	Morning stiffness	+
n = 46/60	Double-blind		2.0 g DHA	Grip strength	++
	Randomized			Walk time	NE
				Tender joints	++
Kremer, 1990	Placebo-controlled	24	27 mg/kg/d EPA	Morning stiffness	++
n = 49/70	Double-blind		18 mg/kg/d DHA	Onset fatigue	+
	Randomized		54 mg/kg/d EPA	Grip strength	++
	Prospective		36 mg/kg/d DHA	Tender joints	++
				Swollen joints	+
Van Tempel, 1990	Placebo-controlled	12	2.04 g EPA	Morning stiffness	++
n = 14/16	Double-blind		1.32 g DHA	Tender joints	NE
	Randomized			Swollen joints	+
	Crossover			Grip strength	NE
Skoldstam, 1992	Placebo-controlled	24	1.8 g EPA	Grip strength	+
n = 43/46	Double-blind		1.2 g DHA	Ritchie index	+
	Randomized			Doctor/global/evaluation	NE
	Noncrossover			NSAIDs reduction	+
Nielsen, 1992	Placebo-controlled	12	2.0 g EPA	Morning stiffness	++
n = 51/57	Double-blind		1.2 g DHA	Grip strength	+
	Randomized			Tender joints	+
				NSAIDs reduction	+

TABLE 22.2 (CONTINUED)
Comparison of the Effects of n-3 Fatty Acid Supplementation on Clinical Variables

Espersen, 1992	Placebo-controlled	12	2.0 g EPA	Ritchie index	+
n = 32/32	Double-blind		1.2 g DHA		
	Randomized				
	Prospective				
Kjeldsen-Kragh, 1992	Placebo-controlled	16	0.54 g EPA	Morning stiffness	+
n = 67/79	Double-blind		0.28 g DHA	Doctor/global/evaluation	+
	Randomized			Patient/global/evaluation	+
Lau, 1993	Placebo-controlled	52	1.71 g EPA	Morning stiffness	NE
n = 37/64	Double-blind		1.14 g DHA	Grip strength	NE
	Randomized			Pain	NE
				NSAIDs reduction	+
Guesens, 1994	Placebo-controlled	52	2.6 g EPA/DHA	Morning stiffness	NE
n = 60/90	Double-blind			Grip strength	+
	Randomized			Ritchie index	NE
				Doctor/global disease	+
				Patient pain assessment	+
Kremer, 1995	Placebo-controlled	48	130 mg/kg/day	Tender joint count	+
n = 41/66	Double-blind		44% EPA	Doctor/global/evaluation	+
	Randomized		24% DHA	Patient/global/evaluation	+
	Prospective			Morning stiffness	+
				Pain	+

[a] ++, highly significant improvement; +, significant improvement; NE, no effect.

Despite the development of a strict protocol for RA supplementation trials, worsening disease activity necessitates a change in treatment. This was usually a predetermined withdrawal criterion. A high withdrawal rate often made statistically meaningful comparisons difficult in many studies, as indicated in Table 22.2. In 1995, the ACR established a preliminary definition for improvement in RA studies.[100] The ACR-20 criteria require subjects to exhibit 20% or greater improvement in tender and swollen joint counts and 20% or greater improvement in three of five clinical variables of patient assessment of pain, patient and physician assessment of global arthritis activity, patient self-assessed disability, and ESR or CRP.

Clinical studies involving n-3 fatty acid supplementation have been conducted with subjects maintaining concurrent pharmacological therapies. Thus, n-3 fatty acid supplementation anti-inflammatory effect is likely to be in addition to the pharmacological effect, thus masking the full potential of n-3 fatty acid supplementation. Clinical trials involving drugs require all other medications to be discontinued. It is in this scenario that the ACR-20 is then applied. In addition to these difficulties, subjects with worsening disease activity are required to change their medication regime. This is usually a predetermined criterion for withdrawal from a study. Tables 22.2 and 22.3 provide evidence of the high withdrawal rates in RA clinical studies.

Kremer has been in the forefront of dietary supplementation trials in RA. In 1990, Kremer's group[16] used two doses of fish oil over 24 weeks in a double-blind, placebo-controlled study. The higher dose of fish oil produced significant clinical changes for tender joint count (TJC), swollen joint count (SJC), early morning stiffness (EMS), and grip strength at 18 weeks ($p < 0.04$), whereas the low-dose fish oil produced significant changes after 24 weeks ($p < 0.05$) (Tables 22.2 and 22.3). The magnitude of these changes was not different between groups at 24 weeks, which suggests that the dose–response determined the time to improvement rather than the magnitude of improvement. In 1995, Kremer's[17] group examined the supplementation effect of dietary fish oil in which NSAIDs were withdrawn in a double-blinded manner. They examined TJC ($p < 0.0001$), EMS ($p = 0.008$), physician and patient global assessment of

TABLE 22.3
Comparison of Effects of n-3 Fatty Acid Supplementation on Biochemical Variables

Ref.	Study Design	Duration, weeks	Amount n-3	Biochemical Parameters	Effect[a]
Kremer, 1985, medication continued	Placebo-controlled Double-blind Randomized Prospective	12	1.8 g EPA	None observed	
Kremer, 1987, medication continued	Placebo-controlled Double-blind Randomized Prospective	2 × 14	2.7 g EPA 1.8 g DHA	LTB_4 LTB_5	++ +
Sperling, 1987, medication continued	Prospective Blind	6	3.6 g EPA 2.4 g DHA	LTB_4 PAF	+ +
Cleland, 1988, medication continued	Placebo-controlled Double-blind Randomized Prospective	12	3.2 g EPA 2.0 g DHA	LTB_4 LTB_5 Combined	++ NE NE
Van Tempel, 1990, medication continued	Placebo-controlled Double-blind Randomized Crossover Prospective	12	2.04 g EPA 1.32 g DHA	LTB_4 LTB_5	NE NE
Skoldstam, 1992, medication continued	Placebo-controlled Double-blind Randomized	24	1.8 g EPA 1.2 g DHA	ERS CRP Platelet count	+ NE NE
Nielsen, 1992, medication change — withdraw	Placebo-controlled Double-blind Randomized	12	2.0 g EPA 1.2 g DHA	ESR CRP	NE +
Espersen, 1992, medication continued	Placebo-controlled Double-blind Randomized Prospective	12	2.0 g EPA 1.2 g DHA	IL-1β TNFα	+ NE
Kjeldsen-Kragh, 1992, medication continued	Placebo-controlled Double-blind Randomized	16	0.54 g EPA 0.28 g DHA	ESR CRP	IN IN
Lau, 1993, change in NSAID end point	Placebo-controlled Double-blind	52	1.71 g EPA 1.14 g DHA	None observed	
Guesens, 1994, varied as required during study	Placebo-controlled Double-blind Randomized	52	2.6 g EPA/DHA	ESR	NE
Kremer, 1995, medication continued, NSAID stopped at 18/22 weeks	Placebo-controlled Double-blind Randomized Prospective	48	130 mg/kg/day 44% EPA 24% DHA	IL-β	+

[a] ++, highly significant improvement; +, significant improvement; IN, increased; NE, no effect.

arthritis activity (PHY-G) (p = 0.017) and (PT-G) (p = 0.036), VAS pain (p = 0.004) in the treatment group for within-group improvement from baseline to the end of the study, while the control group did not show any significant treatment effect. The investigators did observe, however, that 8 weeks after discontinuing NSAIDs, the TJC of the treatment group was significantly lower than the control group. This group ingested 7.1 g daily of n-3 fatty acids, which

TABLE 22.4
Comparison of Clinical Variables in n-3 Fatty Acid Supplementation Studies

Within-Group Differences Treatment Variable[a]	Kremer 1995[17], Mean ± SEM	*n* = 20 *P* value	Volker 1999[101] Mean ± SEM	*n* = 13 *p* value
TJC	–5.3 ±0.8	0.0001	–2.16 ±1.3	0.309
SJC	NA	NA	–4.54 ±1.1	0.007
EMS (mins)	–67.7 ±23.2	0.008	–5.81 ±3.6	0.003
VAS	-0.38 ±0.12 [2]	0.004	–1.94 ±0.6	0.014[3]
PHY-G	–0.33 ±0.13	0.017	–1.58 ±0.4	0.005
PT-G	–0.38 ±0.17	0.036	–2.35 ±0.6	0.018
HAQ	NA	NA	–2.69 ±0.9	0.008
Within-group differences (control)	No significant differences 0–20/22 wks		No significant differences 0–15 wks	
Between-group differences	No significant differences 0–18/22 wks		EMS HAQ	0.017 0.05
RA criteria	OMERACT	Treatment 7/20 Control 1/21	ACR-20	Treatment 5/13 Control 3/13

[a] Values are expressed as mean ±SEM of percent change from baseline to end of study.
[b] Pain reported as physician evaluation of pain.
[c] Pain reported as patient evaluation of pain.

Comment: Kremer et al. (1995) reported changes induced by dietary fish oil supplementation evaluations from baseline to maximum duration of NSAIDs and DMARDs at week 18 or 22.

This study reported changes induced by dietary fish oil supplementation evaluations from baseline to maximum duration of NSAIDs and DMARDs at week 15. Within-group differences refer to changes from baseline to end of study for either treatment and control groups. Between-group differences are measured at end of study between the treatment and control groups.

had little effect on the magnitude of clinical responses. This suggested that fish oil had a modest NSAID sparing effect and that more was not better; that is, beneficial effects could be observed at lower doses.

The results of the study conducted by our group, when compared with the 1995 Kremer study (summarized in Table 22.4) suggests that despite the 48% withdrawal rate, reduction of n-6 fatty acids in the background diet to <10 g/day produced significant results. The quality of life measures of EMS, PT-G, and HAQ suggest that the cohort of subjects in the study conducted by our group experienced significant improvements in health after n-3 fatty acid supplementation.[101]

Most outcome measures in RA supplementation studies have been related to synovitis, in particular the symptoms of joint pain, swelling, tenderness, early morning stiffness, grip strength, and patient/physician global assessment of arthritis activity. These measures of inflammatory activity may be eicosanoid related through the activity of leukotriene B_4 and B_5 (LTB_4/LTB_5) and prostaglandins E_2 and E_3 (PGE_2/PGE_3).[84] Cytokine activity may mediate the later destructive phase of RA, as a result of the activity of interleukins (IL-1, IL-6, IL-8, and IL-10) as well as TNF-α.[102–106] Biochemical variables were assessed in 7 of the 13 studies in Table 22.3, four out of five studies reported significantly reduced levels of LTB_4 after fish oil supplementation, one study reported a significant reduction in platelet activating factor (PAF),[97] while two of three studies reported a significant reduction in IL-1.[16,95] TNF-α was detected in one study,[94] but levels were not significantly changed by fish oil supplementation. Fish oil suppresses cytokine production; hence, fish oil may have a more-pronounced effect on joint destruction than on synovitis. Unfortunately joint destruction was not examined in these clinical studies.[107]

V. EICOSANOIDS

A. Eicosanoid Metabolism

The 20-carbon n-6 and n-3 fatty acids AA and EPA are biosynthetic precursors for the n-6 and n-3 eicosanoids. The biosynthesis of eicosanoids is illustrated in Figure 22.1. Eicosanoids derived by oxidative reactions in the cyclo-oxygenase (COX) and lipoxygenase (LOX) systems are the prostanoids of the 1, 2, and 3 series (PG), thromboxanes (TX), leukotrienes (LT), lipoxins (LX), and various hydroxy acids such as hydroperoxyeicosatetraenoic (HPETEs) and hydroxyeicosatetraenoic (HETEs) acids (Figure 22.1). In most cases, AA is the principal precursor and the eicosanoids derived from AA possess more biologically potent functions than those released from EPA. The precursor PUFA is released from membrane phosphatidylcholine (PC) by the action of phospholipase A_2 or from membrane phosphatidylinositol 4,5-biphosphate (PIP_2) by the action of phospholipase C and diacylglycerol (DAG) lipase.[108,109]

The mechanism of competition between n-6 fatty acids and n-3 fatty acids for the various enzymes of the COX and LOX systems illustrated in Figures 22.1 and 22.2 is summarized as follows:

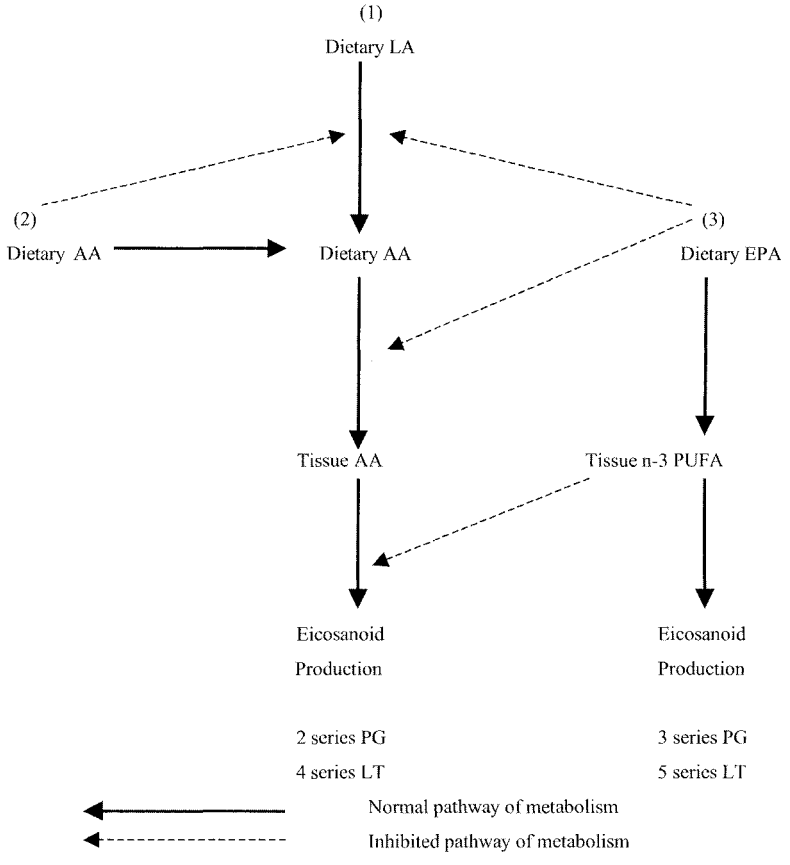


FIGURE 22.2 Regulation of eicosanoid production, illustrating the effects of dietary LA, AA, and EPA on eicosanoid production. (1) AA is derived from LA when it is the sole n-6 PUFA in the diet. This product is then regulated by feedback mechanism when AA accumulates. (2) AA is then incorporated into tissue phospholipids and AA is released for eicosanoid production. (3) When EPA increases in the diet, LA conversion to AA decreases and EPA replaces AA in tissue phospholipids. Enrichment of tissue phospholipids with EPA inhibits AA-derived eicosanoid production. This also results in an increase in EPA-derived eicosanoids.

1. Omega-3 inhibits n-6 desaturase activity to inhibit AA synthesis.
2. Omega-3 fatty acids compete with AA for the sn-2 position in membrane phospholipids, which subsequently reduces plasma and cellular levels of AA.
3. EPA competes with AA as the substrate for the COX system, inhibiting the production of thromboxane A_2 (TXA_2) by platelets.
4. In endothelial cells, prostaglandin I_3 (PGI_3) is synthesized from EPA, which adds to the effect of prostaglandin I_2 (PGI_2) (Figure 22.2 and Table 22.5).

B. Effect of n-3 Fatty Acids on Eicosanoid Metabolism

The eicosanoid metabolic pathway begins with either the COX pathway to produce PGs and TXs or the LOX-5, -12, -15 enzyme system to yield LTs, HPETEs, HETEs, and LXs (Figure 22.1). The quantity of eicosanoid and the precursor used are determined by the availability of AA through the action of phospholipase A_2 and phospholipase C and the activity of the COX and LOX systems. The most biologically active products of the COX pathway are the 2 series PGs (PGA_2, PGE_2, PGI_2, PGF_2, and TXA_2) (Figures 22.1 and 22.2 and Table 22.5), which are produced in a cell specific manner, acting locally, with a short half-life.[109] Production of the 2 series prostaglandins and prostacyclins is initiated by a specific stimuli, such as cytokine, growth factor, endotoxin, oxygen free radical, antigen-antibody complex, bradykinin, collagen or thrombin.[110,111] Some prostanoids have opposite effects; for example, TXA_2 increases platelet aggregation, whereas PGI_2 inhibits platelet aggregation. The prostaglandins are associated with the inflammatory response, as well as the COX inhibitors such as NSAIDs.[112–114]

VI. INFLAMMATION AND THE ROLE OF CYTOKINES IN RA

Cytokines mediate protective immune responses and may be responsible for harmful tissue destruction when produced in excessive amounts.[115] They are soluble proteins, produced by cells as a

TABLE 22.5
Biological Effects of Eicosanoids Derived from Arachidonic Acid and Eicosapentaenoic Acid

Biological Effect	AA C20:4(n-6)	EPA C20:5(n-3)
Thromboxane in platelets	A_2	A_3
	Aggregration	Antiaggregration
	Vasoconstriction	Inactive?
Prostaglandins in endothelial cells	I_2	I_3
	Anti-aggregratory	Antiaggregratory
	Vasodilation	Vasodilation
Prostaglandins in several cells	E_2	E_3
	Immunosuppression	Immunosuppression
	Vasodilation	Inactive?
	Permeability	
	Hyperalgesia	
Leukotrienes in leucocytes	B_4	B_5
	Chemotaxis ×3	Chemotaxis × 1
	Aggregration ×3	Aggregration × 1
	Receptor binding ×3	Receptor binding × 1
	Permeability ×3	Permeability × 1

Note: ×3 denotes biological effect; subscript 2 indicates 2 series eicosanoids; subscript 3 denotes 3 series eicosanoids.

result of activation by specific stimuli. Cytokines influence the activity of cells which express receptors to which they can bind. In binding to these receptors, cytokines are capable of acting in both a paracrine and autocrine manner.[115]

The proinflammatory cytokines that have been identified to date are TNF-α, IL-1, and IL-6. TNF-α is produced mainly by activated neutrophils, monocytes, and macrophages to initiate bacterial and tumor cell killing, to increase adhesion molecule expression, to stimulate T and B cell function, to upregulate MHC antigens, and to initiate the production of IL-1 and IL-6 (Table 22.6). TNF-α is the important link between the specific immune response and inflammation, through its action on both natural and acquired immunity. The production of TNF-α is beneficial in inflammation that is self-limiting; however, overproduction (as is the case in RA) can be problematic because of TNF-α involvement in endotoxic shock, adult respiratory stress syndrome, and other inflammatory conditions.[119] IL-1 shares many of the proinflammatory effects of TNF-α.[116] It is produced mainly by activated monocytes and macrophages. There are two forms of IL-1, alpha and beta, which have similar biological activities and share cell surface receptors.[120] Thus, in the following discussion they will be referred to collectively as IL-1. IL-1 stimulates T and B lymphocyte proliferation and the release of other cytokines such as IL-2 and IL-6, as well as induces hypotension, fever, weight loss, neutrophilia, and acute-phase response. IL-6 is produced mainly by activated monocytes and macrophages in response to IL-1 and TNF-α. IL-6 modulates T and B lymphocyte function and shares many of the TNF-α and IL-1 functions (Table 22.6).[121]

A large number of human studies have investigated the effect of n-3 supplementation on cytokine production in healthy subjects.[122–130] Results from a study of patients with RA indicated that n-3 fatty acids reduce the *ex vivo* production of IL-1 and TNF-α and that cytokine production remained suppressed for a number of weeks after supplementation.[16] Two research groups reported that the n-3 fatty acid effect varied according to the strength and type of stimulus used to induce cytokine production.[123,131] Cooper's group observed that low concentrations of stimulants were more effective than high concentrations of stimulants.[119] Harris and co-workers[132] report increased nitric oxide (NO) metabolites in supplemented volunteers. They concluded that n-3 fatty acids enhanced NO production from endothelial cells.

Clearly, n-3 fatty acids do influence the functional activities of cells of the immune system, although a number of conflicting observations have been made. These fatty acids appear to alter

TABLE 22.6
Biological Effects of Proinflammatory Cytokines TNF-α, IL-1 and IL-6

Biological Effects	TNF-α	IL-1	IL-6
Induction of fever	++	++	+
Production of acute-phase proteins	+	+	++
Activation of T and B lymphocytes	++	+	++
Activation of hematopoiesis	–	+	+
Induction of phosphoipase A_2 and cyclo-oxygenase	+	+	–
Activation of endothelial cells	+	+	–
Activation of synovial cells	++	+	–
Inhibition of proteoglycan synthesis in cartilage	+	+	–
Activation of ossteoclasts	+	+	–
Induction of hypotension	+	+	–
Production enhanced by oxidants	+	+	+
Induction of IL-1, IL-6, IL-8, TNF-α production	+	+	–

Note: + denotes biological effect; – denotes absence of biological effect.

the production of mediators involved in communications between cells of the immune system (eicosanoid, cytokines, NO).[133] They also appear to alter the expression of key cell surface molecules involved in direct cell-to-cell contact (adhesion molecules).[134,135] The production of cytokines and NO is regulated by eicosanoids and therefore n-3 fatty acid–induced changes in the amount and types of eicosanoids produced could partly explain the effects of n-3 fatty acids. It is clear that the effects are exerted in an eicosanoid–dependent manner.

Since TNF-α is an important inducer of IL-1, IL-1 production could be blocked if TNF-α was inactivated. Anti-TNF-α antibodies inhibited the production of IL-1 as well as other proinflammatory cytokines such as granulocyte-macrophage colony–stimulating factor (GM-CFS) which activates mature monocytes and macrophages.[136] This cytokine also induces and maintains HLA class II expression on RA synovial cells in addition to regulating myelopoiesis.[137] Other cytokines that are induced by TNF-α are IL-6, IL-8, and IL-10. The role of TNF-α in upregulating the production of other proinflammatory cytokines is not always a one-way process. If IL-1 in the RA cell joint is blocked using recombinant IL-1 receptor antagonist (IL-1ra), TNF-α in joint cell cultures does not diminish, but downregulates IL-6 and IL-8 production. TNF-α appears to be the cytokine at the apex of the proinflammatory cytokine cascade. [138]

VII. FOOD SOURCES, FUNCTIONAL FOODS, AND SUPPLEMENTATION STRATEGIES

The dietary imbalance between n-3 and n-6 fatty acids is primarily due to a high intake of vegetable oils and a limited intake of fish and other seafood. The correction of this dietary imbalance may be achieved by the use of encapsulated fish oil, dietary fortification with n-3 PUFA, or an LNA-rich diet in combination with a background diet low in LA.[139–141] Dietary manipulation can be a process of selective intake and supplementation. In selective intake, foods that are rich sources of n-6 fatty acids, such as safflower, sunflower, corn, cottonseed, peanut, and blended oils, as well as spreads derived from these oils, should be replaced with canola-based spreads and canola oil (Table 22.7). Dietary supplementation of n-3 fatty acids can be achieved through fish oil capsules and increasing intake of n-3 fatty acid–rich foods. Results from clinical studies by our group suggest that the level of supplementation should be calculated as 40 mg of n-3 fatty acids/kg body weight/day. This level of n-3 fatty acid supplementation with a background diet <10 g/day, was sufficient to achieve cellular incorporation of EPA.[101] Foods rich in n-3 fatty acids are listed in Table 22.7.[142]

VIII. CONCLUSION

Evidence from these clinical and biochemical studies suggests that n-3 PUFA derived from fish oil does have a modest beneficial effect; however, rheumatologists are unwilling to recommend fish oil supplements to their patients with RA because of the confusion surrounding the amount to prescribe, the clinical effectiveness and efficacy, and the additional expense of an unproven remedy. There is a need (1) to define better the overall therapeutic effect of fish oil, (2) to place these dietary supplements in the appropriate niche in relation to NSAID and DMARD, (3) to provide a formula relating to the balance of n-3 fatty acid–rich foods (and n-6-poor foods) to include in the diet and the n-6 fatty acid–rich foods to exclude, (4) to determine the level of fish oil supplementation related to body weight and concentration of EPA and DHA in a supplement, (5) to monitor background diet for n-6 competition at the cellular level, and (6) to determine the critical variables that modulate the effects of dietary supplementation of n-3 fatty acids on the mechanism of inflammation.

TABLE 22.7
List of n-3 Fatty Acid–Rich Food Sources

Food	Serving Size (g)	Total n-3 (g)
Fresh, raw salmon	100	3.0
Fresh, raw tuna	100	1.17
Canned salmon	100	1.4
Canned sardines	100	1.74
Canned mackerel	100	1.4
Canned tuna	100	0.2
Omega eggs[a]	60	0.34
Regular eggs	60	0.06
Flaxseed oil	20	11.0
Gold n Canola oil[a]	20	2.0
Walnut oil	20	1.6
Wheatgerm oil	20	1.4
Soybean oil	20	1.4
Gold n Canola spread[a]	5	0.2
Gold n Canola lite[a]	5	0.15
Butter Canola blend[a]	5	0.12
Linseeds	5	0.9
Walnuts	15	0.75
Pecans	15	0.16

[a] Functional foods.

Source: Sinclair, A. et al., *Austr. J. Nutr. Diet.*, 55: 116–120, 1998. With permission.

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23 Sphingolipids: Mechanism-Based Inhibitors of Carcinogenesis Produced by Animals, Plants, and Other Organisms

Alfred H. Merrill, Jr. and Eva-Marie Schmelz

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I. INTRODUCTION

All eukaryotes and some prokaryotes and viruses contain lipids characterized by a "ceramide" backbone comprising a sphingoid base and an amide-linked fatty acid, to which various head groups are added (Figure 23.1).[1,2] The structures of sphingolipids vary considerably among

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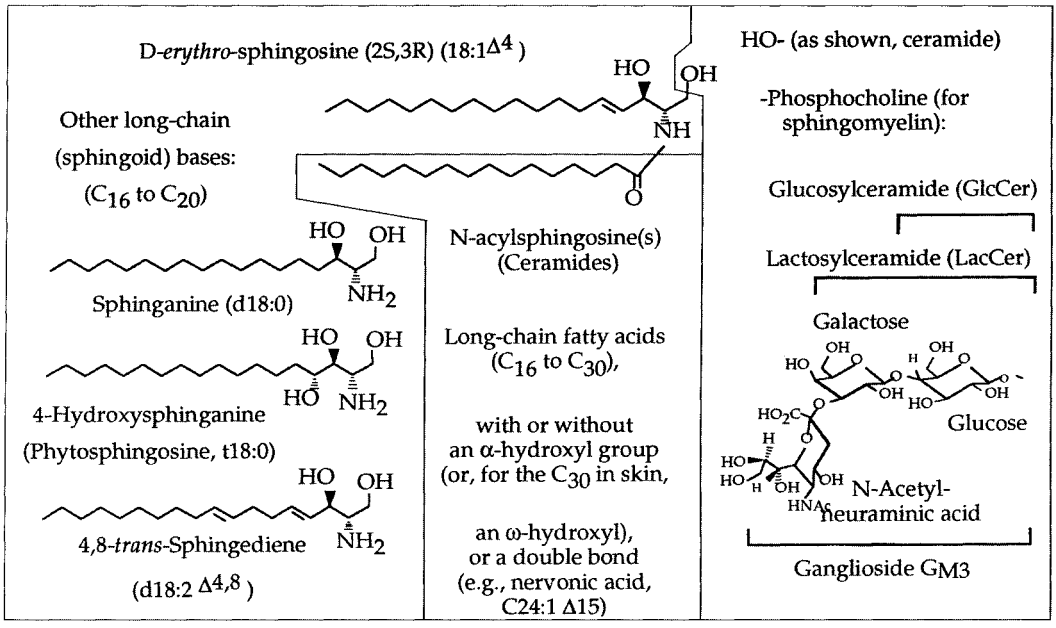

FIGURE 23.1 Structures of representative sphingolipids. Shown are examples of the sphingoid base backbones (named for their di- or trihydroxyl groups, the number of carbon atoms and double bonds, with the position of the *trans*-double bonds designated Δ4 etc.), fatty acids, and head groups.

organisms. Examples are yeast, which have mainly 4-hydroxy-sphingoid bases ("phytosphingosines") and phosphoinositol as the head group; plants and fungi, which have predominantly 4,8-sphingediene and simple hexoses (such as glucose); and mammals, which have mostly sphingosine (4-sphingenine) with both phospho- and simple to complex glyco head groups.[2] The amide-linked fatty acids also vary in size and complexity, from myristic and palmitic acids (C14:0 and C16:0) to α-hydroxy fatty acids, very long chain saturated (e.g., C22:0, C24:0, and C26:0) and monounsaturated (C24:1) fatty acids, and a 30-carbon, ω-hydroxy-fatty acid (in skin ceramides).[3] All together, sphingolipids may be the most structurally diverse category of lipids in nature.

Sphingolipids are primarily components of cell membranes[1]; however, they also appear in lipoproteins,[4] milkfat globule membranes,[5] the lamellar permeability barrier of skin,[3] and other structures. The amounts vary considerably, with the highest proportions being in membrane-rich tissues such as pancreas (which has more sphingomyelin than myelin), liver, neuronal tissues, etc. They are also present in relatively high amounts in some foods, such as dairy products[5] and soybeans[6] (which contain more gangliosides and glucosylcerebrosides, respectively, than brain).[2,5] This latter point is worth noting because the sphingolipids are commonly assumed to be present mainly in neuronal tissues.

There is no known nutritional requirement for sphingolipids.[2] Nonetheless, studies with experimental animals have shown that consumption of sphingolipids inhibits colon carcinogenesis,[7–10] reduces serum low-density lipoprotein cholesterol, and elevates high-density lipoproteins,[11,12] which suggest that they are "functional" components of food. Sphingolipids are also associated with a wide spectrum of biochemical processes that could involve them in other interrelationships between diet and health (for recent reviews, see References 2 and 13). This chapter focuses on the biochemical functions of sphingolipids and the *in vivo* feeding studies that suggest that sphingolipids reduce colon cancer risk and may be protective for other forms of cancer.

II. SPHINGOLIPIDS AND CELL SIGNALING

Sphingolipids influence membrane structure and dynamics and modify the behavior of membrane proteins, including growth factor receptors and matrix proteins.[14] These events occur primarily on the extracellular leaflet of the cell surface, but since the discovery in the mid-1980s that sphingoid bases are potent inhibitors of protein kinase C,[15] an additional paradigm has evolved: that the sphingolipid backbone(s) serve as signaling molecules. While much remains to be learned about these pathways, ceramides, sphingoid bases, and sphingoid base 1-phosphates (and perhaps other metabolites such as *N*-methyl-sphingoid bases and lysosphingolipids) have been reported to mediate cellular responses to growth and differentiation factors, cytokines, 1α,25-dihydroxy-vitamin D_3, and a growing list of toxins and toxic insults (including chemotherapeutic agents and γ-radiation) (see References 13 and 16 through 20). A scheme that outlines this sphingolipid signaling pathway is shown in Figure 23.2 (it also shows how exogenously supplied sphingolipids can affect cell signaling).

Some agonists (such as platelet-derived growth factor, PDGF) activate sphingomyelinase(s) to hydrolyze sphingomyelin to ceramide, which is further metabolized by ceramidase(s) to sphingosine and (via sphingosine kinase) to sphingosine 1-phosphate, which is generally mitogenic and anti-apoptotic.[21,22] Although the downstream targets of sphingosine 1-phosphate are not known, they appear to include release of calcium from intracellular stores and activation of transcription factors, as summarized in References 19 and 23. Sphingosine 1-phosphate can also serve as an extracellular

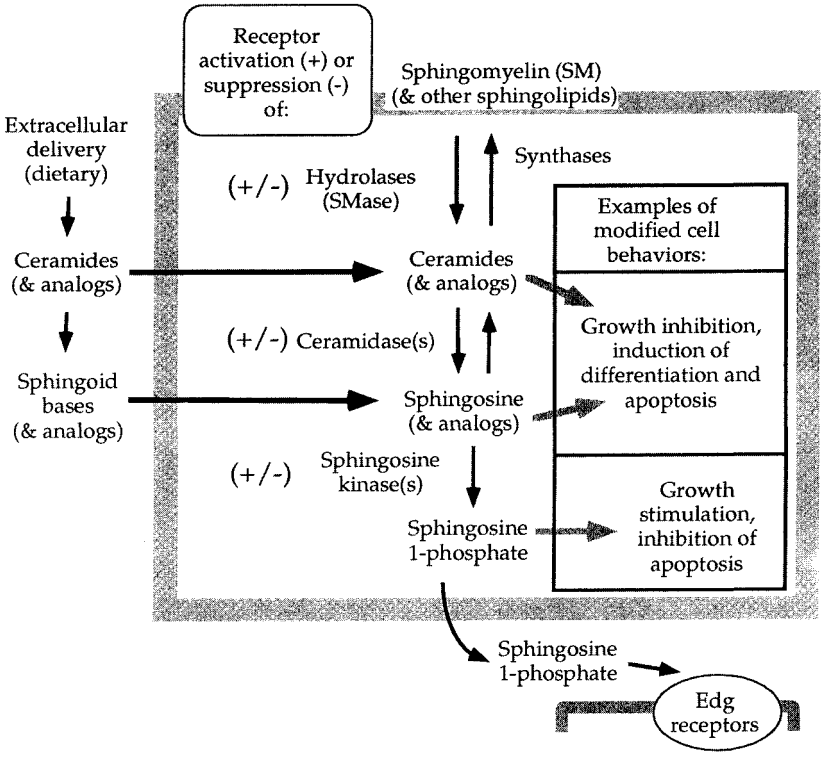

FIGURE 23.2 Sphingolipid metabolism and cell signaling. Shown are the major pathways for metabolism of bioactive sphingolipids as well as some of the cellular behaviors that are affected by ceramide, sphingosine, and sphingosine 1-phosphate. Agonists (such as growth factors and cytokines) induce the activation (or suppression) of the hydrolytic enzymes of these pathways to alter the amounts of these compounds. Cell behavior can also be affected by delivery of the compounds to the cells from an exogenous source, such as the diet. Some cells release sphingosine 1-phosphate which activates Edg family receptors on endothelial cells.

messenger.[20] In contrast, growth inhibitory and cytotoxic agonists (such as tumor necrosis factor-α, TNF-α) usually activate sphingomyelinase(s) but not sphingosine kinase; thus, ceramide (and perhaps sphingosine) accumulates and inhibits growth and induces apoptosis.[14,24–26] Thus, the formation of sphingosine 1-phosphate vs. ceramide and/or sphingosine can determine whether a given cell stimulant causes growth or death.

III. SPHINGOLIPIDS AND CANCER

Cancer involves numerous defects in cell regulation and, as will be described below, sphingolipids have been found to affect almost every aspect of defective cell regulation in cancer (Table 23.1).

A. Sphingolipids and the Behavior of Transformed Cells in Culture

Sphingosine and ceramide are growth inhibitory and toxic for many transformed cells lines (HL60, Jurkatt, HT29, and CHO cells, *inter alia*) at relatively low concentrations (i.e., below the concentrations where they have nonspecific detergent effects).[27] The evidence to date suggests that sphingoid bases and ceramides inhibit growth by increasing the expression of inhibitors of cyclin-dependent protein kinases,[28,29] and induce cell death via activation of caspases and apoptosis (although other pathways, including necrotic cell death, have not been ruled out). Furthermore, sphingoid bases and ceramides are not only effective individually, but may be more potent when used together.[26]

Sphingolipids are also able to suppress very early events in cell transformation.[30,31] For these studies, a relatively "normal" cell line (C3H10T1/2 cells) that normally undergoes density-dependent growth arrest was treated with a chemical carcinogen or γ-irradiation followed by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) as a "tumor promoter" to produce cells that escape growth arrest and appear as visible foci. Addition of sphingosine or sphinganine greatly reduced the number of transformed foci with and without "promotion" by TPA.[30,31] These effects were associated with inhibition of protein kinase C; however, this did not appear to be the only mechanism whereby sphingolipids suppressed early events in carcinogenesis.[30,31]

B. Sphingolipids and Skin Carcinogenesis

In extending these findings to *in vivo* models for cancer, the first complication is delivery of lipophilic sphingolipids to the target cells. This difficulty can be circumvented in some cases by studying skin, where topical administration is feasible. The efficacies of sphingosine, *N*-methylsphingosine, and *N*-acetylsphingosine were assessed in female Sencar mice by measuring the induction of epidermal ornithine decarboxylase activity and hyperplasia by TPA.[32,33] Induction of ornithine decarboxylase was inhibited by all of these compounds, with sphingosine being the most potent; however, hyperplasia was not inhibited.[32–34]

TABLE 23.1
Processes of Relevance to Cancer That Can Be Modulated by Sphingolipids

Defects in Cell Regulation in Cancer	Cell Behaviors Modified by Sphingolipids
Hyperproliferation	Growth inhibition[a]
Loss of differentiation	Induction of differentiation
Inhibition of apotosis	Induction of apotosis[a]
Acquisition of multidrug resistance	Inhibition of multidrug resistance
Increased cell migration (metastasis)	Inhibition of cell migration and metastasis
Angiogenesis	Inhibition of angiogenisis[a]

[a] Effects of adding sphingosine and/or ceramide or modifying their metabolism; sphingosine 1-phosphate usually has the opposite effect.

Skin cancer was induced by the combination of 7,12-dimethylbenz[a]anthracene (DMBA) and TPA using two application protocols.[34] In the first, the mice were treated with DMBA then given twice weekly applications of TPA (for 15 weeks) and the sphingolipids were applied before each TPA application. Papilloma incidence and multiplicity were not reduced, but *N*-acetylsphingosine and *N*-methylsphingosine increased cancer-free survival. In the second protocol, the sphingolipids were applied before each TPA treatment and twice weekly for 10 weeks after the final TPA treatment. Again, papilloma incidence and multiplicity were not reduced; however, sphingosine and *N*-acetylsphingosine decreased the proportion of mice with carcinoma. Thus, sphingoid bases and ceramides inhibited carcinoma development by both protocols. Nonetheless, at high levels sphingosine sometimes enhanced rather than suppressed tumorigenesis[33,34] and the reason or reasons for this opposite effect are not known. One possibility is that formation of sphingosine 1-phosphate may be involved, and this is discussed later in this chapter.

C. Sphingolipids and Colon Carcinogenesis

Another form of cancer that is accessible via "topical" administration is colon cancer if the agent is not completely absorbed in the upper intestine and can reach colonic cells. This is the case for dietary sphingolipids because they can be digested to ceramide and sphingosine in both the upper and lower intestine (probably with contributions from colonic microflora) and, depending on the nature of the sphingolipid, a significant portion can reach the colon (Figure 23.3).[35–37]

The effects of dietary sphingolipids on colon carcinogenesis have been studied using CF1 mice administered *N,N*-dimethylhydrazine (DMH) then fed diets that contain varying amounts of sphingolipids. The first of these studies used sphingomyelin because it was possible to purify gram amounts of this sphingolipid from an inexpensive and relatively rich source, nonfat dry milk. The controls were fed a standard AIN76A diet, which is composed of defined ingredients (casein, corn

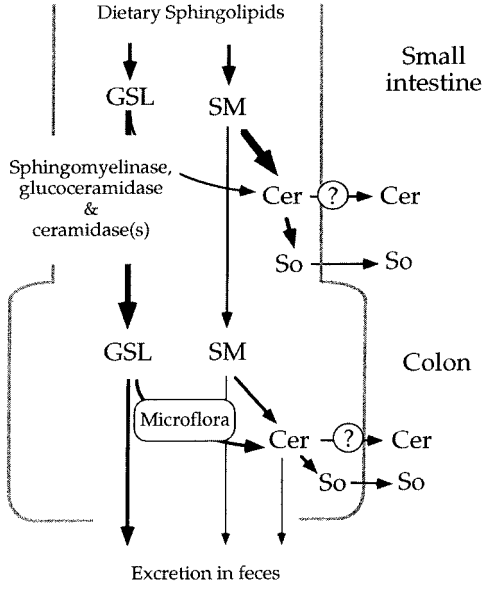

FIGURE 23.3 Diagrammatic representation of the digestion of sphingolipids in the small intestine and colon. In small intestine, sphingomyelin (SM) and simple glycosphingolipids (GSL) such as glucosylceramide are hydrolyzed to ceramide (Cer), which is further cleaved to the sphingoid base (sphingosine, So) that is taken up by intestinal cells. A portion of the SM and GSL (especially GSL with carbohydrate head groups that are poorly hydrolyzed) reach the colon where they are hydrolyzed to Cer and So by enzymes that may be provided by intestinal microflora. Some sphingolipid is excreted.

oil, etc.) that contain very low amounts of sphingolipids. Sphingomyelin supplementation at 0.1% of the diet (wt/wt) reduced the number of aberrant colonic crypt foci (an early marker of colon carcinogenesis) by approximately 70% and, with longer feeding, reduced the number of colonic adenocarcinomas.[7] In a follow-up investigation,[8] sphingomyelin feeding caused a comparable reduction in aberrant colonic crypt foci, the number of crypts per focus, and the number of adenocarcinomas. Interestingly, the reduction in adenocarcinomas in the sphingomyelin-fed mice was counterbalanced by an increase in adenomas in the second study.[8] This raises the possibility that sphingomyelin suppresses the conversion of adenomas to adenocarcinomas.

It warrants comment that the amounts of sphingomyelin used in these experiments (0.025 to 0.1% of the diet, w/w) are near the amounts that are consumed in the United States (0.01 to 0.02% of the diet) (Table 23.2)[2] (and this may underestimate the amounts that are consumed by humans because relatively few foods have been analyzed for sphingolipids). Unfortunately, little is known also about the bioavailability of dietary sphingolipids. The only in-depth studies of sphingolipid digestion, uptake, and elimination have been conducted with rodents, and they suggest that both sphingomyelin and glucosylceramide are digested in the small intestine, but significant amounts reach the colon.[35–37]

D. Possible Mechanisms of Action of Sphingolipids in Suppressing Colon Carcinogenesis

The amounts of dietary sphingolipids that caused these effects on colon carcinogenesis had no obvious deleterious effects[8–10] on the animals, and other studies have not reported toxicity at much higher amounts.[11,12] Hence, why are such small amounts affecting the behavior of transformed colonic cells? One possibility is that exogenously supplied sphingolipids bypass a sphingolipid signaling defect that is important in cancer — for example, a loss of cellular sphingomyelin turnover to produce ceramide and sphingosine. There is some evidence for this hypothesis in both rodent and human colon cancer. Dudeja and colleagues[38] noted that within a day after treatment with *N,N*-dimethylhydrazine, there is a change in membrane fluidity of colonic cells, which they attributed to an increase in sphingomyelin due to a reduction in sphingomyelinase activity and an increase in sphingomyelin synthesis. Hertervig and colleagues[39] have found that sphingomyelinase activity is also decreased in human colorectal carcinoma compared with adjacent healthy tissue. If these findings represent a fundamental defect in cancer — i.e., the loss of sphingomyelin turnover to regulate growth and apoptosis — the supply of ceramide and/or sphingosine via the diet may bypass this defect and restore "normal" cell function. This might explain why dietary sphingolipids are active in relatively low amounts.

A few studies have provided other evidence that abnormal cell behavior can arise from defects in sphingolipid signaling. In one case,[40] a cell line was isolated based on its resistance to apoptosis induced by TNF-α. The defect was shown to be due to loss of sphingomyelin turnover that could be bypassed by addition of ceramide. In another,[41] defective sphingolipid signaling was shown to be a component of the resistance of prostate cancer cell lines to chemotherapeutic agents. A growing number of therapeutic modalities for cancer (both irradiation and chemotherapy) have been found to involve induction of sphingolipid turnover or *de novo* biosynthesis[42–47]; hence, this may be a widespread mechanism in tumor biology.

E. Structure–Function Relationships between Sphingolipids and Their Effects on Colon Carcinogenesis

To exclude the possibility that the effects of milk sphingomyelin are due to a copurifying contaminant, *N*-palmitoylsphingomyelin was synthesized and found to have the same effect on suppression of aberrant colonic crypt foci formation (Figure 23.4).[9] As part of this study, dihydrosphingomyelin (i.e., with a sphinganine backbone) was also tested, and caused an even greater reduction in the

TABLE 23.2
Examples of the Amounts of Sphingolipids in Food and Yearly Sphingolipid Consumption per Capita[a]

Food Sources	Sphingolipid Content, μmol/kg	Sphingolipids Consumed per Capita, μmol/year
Dairy products		
Total		38,464
Milk (3.5%)	160	5,764
Lowfat milk (<2%)	92	5,486
Cheese (29%)	1,326	15,912
Meat products and fish		
Total		34,270
Beef and veal	390	11,310
Chicken	530	11,660
Fish	130	910
Eggs	2,250	31,500
Vegetables		
Total		8,100 to 33,800[b]
Tomato	42	1,722
Potato	69	4,116
Spinach	67	20
Soybeans	2,410	na
Lettuce	50	500
Fruits		
Total		3,200 to 5,400[b]
Apples	69	1,725
Peanuts	78	234
Cereals		
Wheat flour	576	38,016
Total sphingolipid intake (μmol/year)		153,551 to 181,419[b]
Total sphingolipid intake (g, calculated as sphingomyelin)		116 to 139[b]

[a] For the references from which these calculations were made, see Reference 2.
[b] Estimated.

number of aberrant colonic crypt foci (Figure 23.4). This finding is noteworthy because induction of apoptosis by ceramide requires the 4,5-*trans*-double bond[48] that is lacking in dihydrosphingomyelins. Thus, the active metabolite(s) of dietary sphingomyelin is probably not the intact ceramide backbone but rather the free sphingoid base(s).[25–27] However, it will be necessary to determine if dietary dihydroceramides can be converted to ceramides *in vivo* since cells have this capability as part of *de novo* sphingolipid biosynthesis.[49]

Another structural variable is the head group (see Figure 23.1). Milk glycosphingolipids (glucosylceramide, lactosylceramide, and ganglioside G_{D3}) also suppressed aberrant colonic crypt foci formation[10] (Figure 23.4), and ganglioside G_{M1} was four- to eightfold more potent than sphingomyelin in suppression of aberrant colonic crypt formation.[7] All of these categories of glycolipids

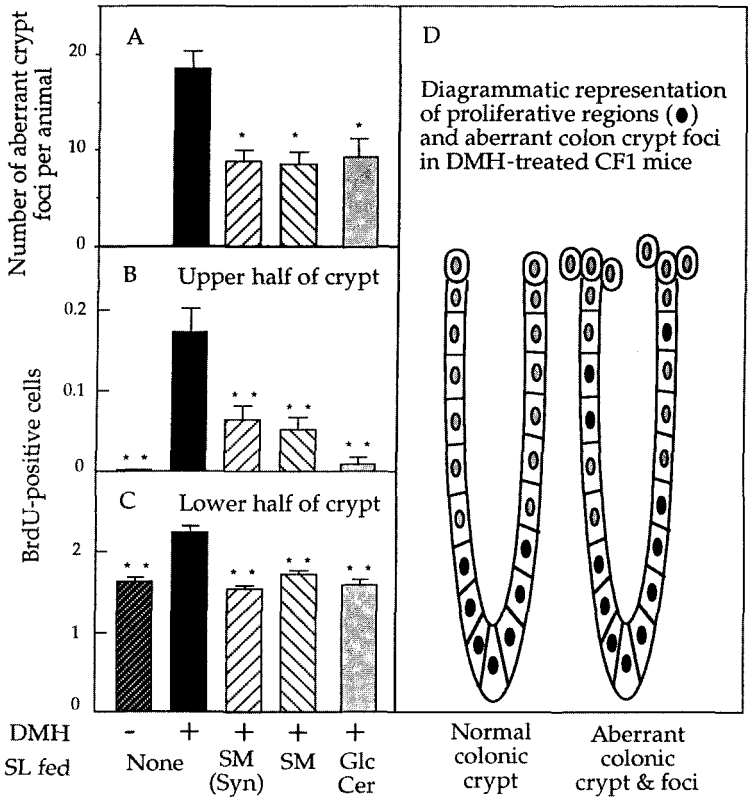

FIGURE 23.4 Effects of dietary sphingolipids on colonic cell proliferation and aberrant colonic crypt formation in CF1 mice treated with *N,N*-dimethylhydrazine. The feeding of synthetic sphingomyelin (SM, Syn) or sphingolipids isolated from dairy sources (sphingomyelin, SM, and glucosylceramide, GlcCer) reduces aberrant colonic crypt formation (A) and colonic cell proliferation (B and C), with the greatest effect on proliferation for cells of the upper crypt in DMH-treated mice (B). (D) Diagrammatic illustration of these changes in colonic crypt cell histology. (Data for this figure are from References 7 through 10.)

are hydrolyzed by enzymes in the lumen of CF1 mice (it is not known if the activities are produced by the mice or intestinal microflora, or both).[10] Hence, it appears that a substantial portion of the sphingolipids that are found in food[2] (see Table 23.2) may be digestible and may provide protection against colon carcinogenesis.

F. Sphingolipids and Human Colon Cancer

No human clinical trials have yet evaluated sphingolipids for the prevention or treatment of colon cancer, nor have there been, as far as we are aware, any epidemiological studies of possible associations between sphingolipid consumption and cancer risk. There are, nonetheless, reasons to believe that sphingolipids will inhibit colon cancer in humans. First, sphingosine and ceramide induce apoptosis in a human adenocarcinoma cell line (HT29 cells)[50] and we have recently found a similar effect with additional human colon cancer lines (SW480, HCT116, and T84) (Schmelz and colleagues, unpublished observation).

Second, we have just completed a study of the effect of feeding sphingolipids to Min mice and a mixture of ceramide and complex sphingolipids (totaling 0.1%, w/w, of the diet) reduced the number of intestinal tumors by 55% (Schmelz and colleagues, manuscript in preparation). Min mice have a genetic defect similar to that found in human familial adenomatous polyposis and

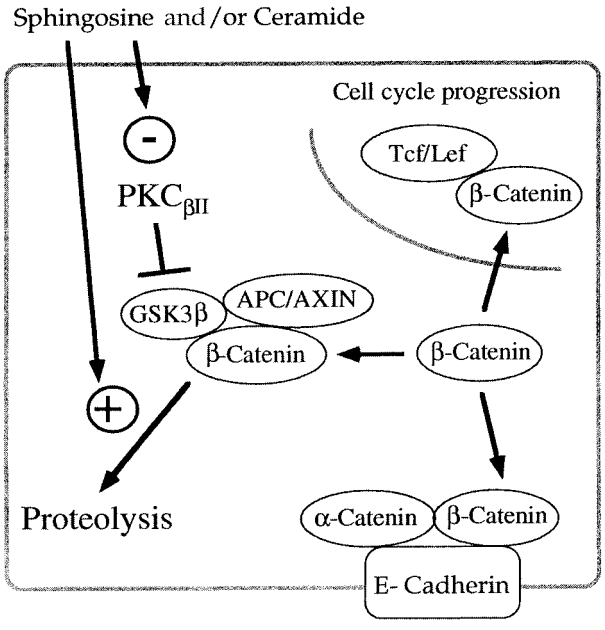

FIGURE 23.5 A chematic representation of the abnormalities in β-catenin behavior in cells with defective APC or other regulators of β-catenin function, and possible mechanisms for the reduction in cytosolic β-catenin in colon cancer cells in culture given sphingosine and Min mice fed sphingolipids. Under normal conditions, β-catenin is mostly associated with E-cadherin or proteolytically degraded (with participation of APC and glycogen synthase kinase, GSK); however, in cells with mutated APC (in Min mice and many human colon cancers) or other regulators of this pathway, β-catenin accumulates in the cytosol and enters the nucleus, where it participates in the induction of genes that cause proliferation (via Tcf/Lef transcription factors). The reduction of cytosolic β-catenin and inhibition of growth of sphingolipids may be via inhibition of protein kinase C (PKC) or effects on other signaling pathways by sphingosine and/or ceramide as described in the text.

which is also found in a large percentage of sporadic human colon cancers.[51] Min mice (and human colon cancer cells with mutations in the APC gene) accumulate β-catenin in the cytosol as shown in Figure 23.5. We find that the Min mice fed sphingolipids (and human colon tumor cell lines treated with sphingosine) have less cytosolic β-catenin, which is consistent with normalization of this signaling defect by sphingolipids, perhaps at the sites shown in Figure 23.5. Thus, sphingolipid consumption can affect a specific genetic mutation in mice that is prototypic of a similar genetic defect in humans.

The third piece of evidence comes from studies of a synthetic sphingoid base analogue (safingol) that has been developed as a potential cancer chemotherapeutic agent, and is discussed in the following section.

G. Cancer Chemotherapy Studies Using L-*threo*-Sphinganine (Safingol)

Safingol is the L-*threo*-isomer of sphinganine, and was developed as an anticancer lead by Sphinx Pharmaceuticals (which is now part of Eli Lilly) based on its ability to inhibit protein kinase C[52] and to have a longer biological lifetime since it is not a substrate for sphingosine kinase.[53] Toxicity studies[54] in dogs administered safingol in single intravenous doses of 5 to 40 mg of safingol/kg found no evidence of adverse systemic toxicity at up to 20 mg/kg; however, escalating doses of 5, 10, 20, 30, 40, and 75 mg/kg resulted in red urine at 10 mg/kg and higher, icterus following 40 mg/kg, and additional signs of hypoactivity and anorexia occurring after 75 mg/kg. Pathology revealed marked hepatotoxicity, venous degeneration and necrosis at injection sites, and evidence

of intravascular hemolysis. Local effects of venous irritation or intravascular hemolysis were observed at 20 and 40 mg/kg.

Schwartz and coinvestigators[55] have conducted a pilot clinical trial with safingol administered as a 1-h infusion at escalating doses (from 15 to 120 mg/m^2). After 14 days, patients received the same dose of safingol in combination with a fixed dose of doxorubicin. The mean plasma half-life for safingol was 4 ± 2.5 h, the mean estimated clearance was 3140 ± 765 ml/min, and the mean volume of distribution was of 995 ± 421 l. Coadministration of doxorubicin did not significantly change the pharmacokinetics of safingol, or vice versa. These authors concluded that safingol can be given safely, and commented that minor responses were observed in three patients with pancreatic cancer and one patient with angiosarcoma of the scalp. Although this is an "unnatural" stereoisomer of sphinganine, it may indicate that dietary sphingolipids can inhibit other types of cancer when delivered in sufficient amounts.

H. Other Studies of Sphingolipids and Cancer

There have been almost 3000 publications on the general topic of "sphingolipids and cancer" and the list of possible mechanisms whereby sphingolipids may affect cancer continues to grow.

1. Multidrug Resistance

A particularly intriguing relationship between sphingolipids and multidrug resistance (MDR) has evolved from the observations[56,57] that MDR cancer cell lines (MCF-7-AdrR, an adriamycin-resistant subclone of MCF-7 cells, and MDR KB-V-1 carcinoma cells) accumulate glucosylceramide. This research group further discovered that tamoxifen, a widely used breast cancer drug, was a potent antagonist of glucosylceramide synthesis, and that clinically relevant concentrations of tamoxifen, verapamil, and cyclosporin A (which all circumvent MDR) markedly decrease glucosylceramide levels in MCF-7-AdrR cells.[58] When MCF-7-AdrR cells were treated with an inhibitor of glucosylceramide synthesis (1-phenyl-2-palmitoylamino-3-morpholino-1-propanol), they recovered sensitivity to adriamycin. The MCF-7-AdrR cells are resistant to ceramide due to an increased capacity to convert ceramide to glucosylceramide, [59,60] which can be reversed by adding inhibitors of the conversion of ceramide to glucosylceramide (tamoxifen or RU486, Mifepristone).[61]

To determine whether there is a cause-and-effect relationship between glucosylceramide synthesis and multidrug resistance, Lui and colleagues[62] introduced glucosylceramide synthase into wild-type MCF-7 breast cancer cells using a retroviral tetracycline-on expression system, and the resulting cell line demonstrated strong resistance to adriamycin and to ceramide vs. the original MCF-7 cells. This finding strongly links abnormal glucosylceramide formation to MDR mechanistically. An association between abnormal glucosylceramide formation and MDR in the tumors was also found[63] in six melanoma specimens and one breast tumor specimen obtained from patients who had undergone chemotherapy; hence, this may be a common characteristic of, and possibly even a marker for, multidrug-resistant tumors.

Sphingoid bases also appear to affect the sensitivity of MDR cells to chemotherapeutic agents because treatment of MCF-7 DOXR cells with safingol increased accumulation of [^{3}H]vinblastine and enhanced the toxicity of *Vinca* alkaloids and anthracyclines.[64] The authors concluded that the enhanced drug accumulation and sensitivity in MCF-7 DOXR cells correlated with inhibition of protein kinase C rather than through direct interaction with *P*-glycoprotein.[64] Sphingosine and *N,N*-dimethylsphingosine (DMS) induce apoptosis in human epidermoid carcinoma KB-3–1 and its MDR subclone[65] (and, as already noted, chemotherapy-resistant prostate cancer cells appear to have aberrant sphingolipid signaling).[41] Hence, whether or not sphingoid bases affect multidrug resistance directly, their ability to circumvent multidrug resistance may provide a new strategy for treating drug-resistant tumors.

2. Sphingolipids and Immunosuppression

Complex glycosphingolipids also have roles in immunosuppression that have been recently discussed.[66,67] This appears to be due to the tendency of tumors to secrete large amounts of glycosphingolipids, which block the response of the immune system to the tumor cells. It may be possible to thwart this immunomodulation if the chemotherapeutic agent suppresses glycolipid synthesis by the tumor, or results in the formation of a different profile of metabolites that are less immunosuppressive.

3. Sphingolipids and Metastasis

The role of sphingolipids in metastasis has focused mainly on the expression of complex sphingolipids as cell adhesion molecules and, for example, the ability of simpler glycolipids (such as α-galactosylceramide) to suppress metastasis.[68] However, inhibition of protein kinase C by sphingosine inhibits invasion of human ovary cancer cells,[69] and sphingoid base analogues inhibit the growth and metastasis of human and mouse tumor cells in athymic and euthymic mice.[70,71] Therefore, sphingolipids may inhibit metastasis by many mechanisms.

I. Utilization of Findings from Naturally Occurring Sphingolipids to Identify More Effective Species (and Vice Versa)

Although these findings with sphingolipids are promising, might it be possible to optimize some of the structural features to increase the efficacy?

1. Optimization of the Sphingoid Base Backbone

The underlying hypothesis for the findings with dietary sphingolipids is that delivery of ceramide and/or sphingosine to transformed cells inhibits growth, induces differentiation, and promotes apoptosis (see Figures 23.2 and 23.3). However, another metabolite of this pathway, sphingosine 1-phosphate, is mitogenic[21,72,73] and can inhibit apoptosis,[22,23] which suggests that when sphingosine (from digestion of dietary sphingolipids) is metabolized to sphingosine 1-phosphate, this will interfere with the effectiveness of the sphingolipids as antitumor agents.

To test this hypothesis, we have compared the effectiveness of a series of 1-deoxy analogues of sphingosine and sphinganine (K. Desai and colleagues, manuscript in preparation) in induction of apoptosis in HT29 cells, and found that removal of the 1-hydroxyl group increases the toxicity by as much as tenfold. Therefore, these findings suggest that decreasing the formation of sphingosine 1-phosphate by using naturally occurring (or synthetic) sphingoid bases that are poor substrates for sphingosine kinase (and/or inhibit this enzyme) could be much more effective inhibitors of carcinogenesis.

2. Optimization of the Sphingolipid Head Group

For these bioactive backbones to affect cancer cells, they must be able to reach the site(s) of action which, for colon cancer, means that the optimal compound(s) will pass unaltered through the upper intestine and be completely hydrolyzed to the backbone(s) in the colon. Such compounds are termed *pro-drugs* and complex sphingolipids (sphingomyelin and glycosphingolipids) apparently serve as pro-drugs because they are only partially digested in the small intestine and "deliver" ceramide and sphingosine to colonic cells.

Conjugation of a bioactive compound with β-glucuronic acid provides an even better pro-drug for the selective delivery of compounds to the colon[74] because there is little or no hydrolysis of β-glucuronides in the upper intestine of mammals (unless the upper intestine is contaminated with microflora, as often occurs in rodents), but many colonic microorganisms produce β-glucuronidases

that can act on such conjugates. We have synthesized the β-glucuronide conjugate of ceramide and demonstrated that it is cleaved by purified *Escherichia coli* β-glucuronidase and by activities extracted from mouse colon.[75] Furthermore, the feeding of ceramide β-glucuronide to CF1 mice after treatment with DMH reduced the number of aberrant colonic crypt foci.[75] Thus, this conjugate, or perhaps others based on the same principle, might be more effective inhibitors of colon cancer.

J. Perspectives for the Future: Sphingolipids as Mechanism-Based Bioceuticals

Much has been learned about the mechanisms of action of sphingolipids in cell signaling and how these pathways may be relevant to human cancer. Much more information is needed before the human impact of these compounds is fully known; however, one can imagine that persons at risk of colon cancer may someday consume diets enriched with naturally occurring sphingolipids to reduce their risk. Use of more potent synthetic analogues might also have chemotherapeutic benefits; hence, this category of compounds might be both chemopreventive and chemotherapeutic. As far as we are aware, no term has been coined to describe compounds that are both "nutraceuticals" and "pharmaceuticals"; perhaps the term *bioceuticals* fits this category.

Although the published studies to date have utilized sphingolipids isolated from mammalian sources, sphingolipids are found in many foods, including plants (see Table 23.2). However, sphingolipids from plants, fungi, and other organisms have sphingoid base backbones distinctively different from those of mammalian sphingolipids (e.g., with 8-*trans*- or 4,8-*trans*-double bonds rather than the 4-*trans*-double bond),[76] which may affect their potency. Nonetheless, we have found that 4,8-sphingediene and the ceramide derived from soybean glucosylceramide induce apoptosis in HT29 cells (E. M. Schmelz and colleagues, manuscript in preparation). Hence, sphingolipids from many dietary sources may be capable of affecting carcinogenesis, and should be examined for their potential to suppress colon cancer and, perhaps, cancers of other organs.

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24 Applications of Herbs to Functional Foods

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I. INTRODUCTION

Functional foods have been defined as foods with ingredients (either naturally occurring or added) that provide a health benefit beyond the traditional nutrient value of the food. To date, however, no regulatory definition of *functional foods* or the similar terms *nutraceuticals* and *designer foods* has been proposed or approved by the Food and Drug Administration (FDA). Herbs or botanicals may have health benefits that are not derived from the nutrient composition of the plant. When these herbs are provided in the form of a capsule, powder, softgel, gelcap, or other form that is not represented as a conventional food, these products are considered dietary supplements, and are regulated much differently from foods or ingredients. With the growing interest in herbal products and an attitude toward wellness in the population, it is only natural that food manufacturers would turn to herbals as a new source of functional ingredients. Herbal additives have begun to appear in conventional foods ranging from teas and juices to snack chips and energy bars. In one of the more creative efforts, Dependable Gift and Candy, Inc. of Thousand Palms, CA markets four different NutraPops™ candy with herbs: Happy has a piña colada flavor with St. John's wort, Smarty has a cappuccino flavor with ginkgo biloba,

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Skinny claims "chocolate indulgence flavor with pyruvate and chromium picolinate," and Sexy has "strawberry cream" flavor with kava kava and passionflower added.[1] In the absence of a separate regulatory category for these and other functional foods, such products are regulated as conventional foods.

This chapter discusses botanicals as functional food ingredients, with the idea that the purported health benefits of the food are due to the botanical. Although other ingredients may also be beneficial, this chapter describes benefits associated with the botanicals. Because botanically enhanced functional foods are relatively new in the marketplace, no data exist regarding the efficacy of herbal compounds delivered in this way. Therefore, this chapter reviews numerous herbal compounds, their claims, and the evidence for their efficacy. In addition, the chapter describes some of the foods that do contain herbal ingredients, and the challenges and uncertainties faced in this new functional food arena.

II. HERBAL MEDICINE

Herbal products are showing resurgence in the United States following the passage of the Dietary Supplements Health and Education Act of 1994 (DSHEA). This act amended the Federal Food, Drug and Cosmetic Act to allow dietary supplements to be regulated differently from conventional foods or drugs. Since the passage of this law, herbal and botanical supplements have shown double-digit growth in sales.

But the dietary supplements of today are a far cry from the herbal medicine practiced in ancient China, Greece, Europe, or even by native Americans. Traditional herbalists used plant roots, leaves, bark, flowers, and seeds to prepare teas, broths, tinctures, poultices, and the like. Today, highly purified extracts and powders are put into capsules and tablets. Modern herbal medicine (or phytotherapy) relies on standardization, and demonstrated efficacy–constituent relationships.[2] Although the anecdotal evidence of tradition supports the efficacy of herbal remedies, few comprehensive studies have been done to support the efficacy of modern herbal medicines.

III. HERBS AS INGREDIENTS IN FUNCTIONAL FOODS

There is wide variation in the level of research available to support claims for many herbal supplements. Some, like ginkgo biloba, have been studied extensively in Europe, while others, such as hawthorn leaves and berries, have not been tested in humans. Even less is known about herbals as food ingredients. When herbal compounds have been tested in clinical trials, it is usually as a standardized extract, or specific supplement, rather than as part of the food.

Numerous issues are raised when considering botanical ingredients as food additives, including regulatory requirements, safety, and identity, in addition to efficacy. One of the biggest questions is the stability of botanical ingredients in foods that must be processed by heat, air, or pressure.

A. Regulatory Status

As mentioned previously, no separate regulatory category exists for functional foods. As such, regulations for conventional foods apply. To be used in a food, an herbal ingredient must be "generally recognized as safe" (GRAS) or approved as a food additive. Herein is one of the important distinctions between dietary supplements and foods. Notification of a new dietary ingredient (not in use prior to October 15, 1994) for a dietary supplement is given to the FDA 75 days before first marketing of the supplement. The FDA then evaluates the safety information provided and accepts or rejects the substance as a new dietary ingredient. For foods, however, if an herbal ingredient is not already GRAS or has a reasonable chance of being affirmed as GRAS, a lengthy food additive petition process is indicated.

The FDA has informally allowed companies to self-assert GRAS status provided notification is given to the FDA, and appropriate safety data are made available to the FDA upon request. However, no final rules have been published to guide this process, and there are no clear standards for judging the data provided by companies. As such, determining which herbs are considered GRAS is not a simple task. Additional regulatory issues such as label statements and claims are discussed later in this section.

B. Identity Dilemma

When considering botanicals as food ingredients, one of the fundamental issues is determining the correct identity of the plant compound. There are two issues with identity that can be documented in the literature. The first case is the problem of harvesting the correct genus of plant. Two individuals were poisoned when the herbal preparation they consumed contained the *Digitalis lanata* plant rather than *Plantain*, of the plantago genus.[3] Both individuals experienced cardiac symptoms as a result of elevated levels of digoxin. After tracing the product back through the manufacturer, supplier, and farmer, it was determined that the identity of the *D. lanata* had been confused for the plantain at harvest.

The other issue concerns identifying the correct species of plant. The efficacy of *Echinacea* cannot be extended to all species: *E. purpurea*, *E. angustifolia*, and *E. pallida*. Studies may be flawed because one species had been studied but another species was reported. This confusion resulted in a denial by the German Commission E for approval for some of the echinacea preparations.[2] The Commission E has approved the oral use of *E. purpurea* herb, i.e., the aboveground parts, for colds, respiratory tract infections, and urinary tract infections and the topical use for poorly healing wounds. The *E. pallida* root (fresh or dried) has been approved for use in the treatment of influenza-like infections. The *E. angustifolia* root/herb and the *E. pallida* herb have not been approved due to the confusion in the identity of the actual plant that was studied.

C. Standardization Dilemma

Standardization of herbal ingredients is another relevant issue for both food manufacturers and consumers. Herbs are grown under different conditions, at different locations, and in different seasons of the year. These geographic and environmental differences result in variations in the levels of active compounds. The community of herbal growers, producers, etc. has called for standardization of herb products. But which compounds should be standardized? In some cases, the active compound is unknown. Even if the active ingredient is known, there are many different active compounds in herbs that may act additively or synergistically. Little is known about the optimum level of these compounds. Much research is needed to determine the appropriate standards for quantity, potency, and content uniformity.

D. Effect of Processing Dilemma

Clearly, many of the active compounds in the herbals that may be used in a nutraceutical are not known. More importantly, with the addition of those herbs to food and the processing that the food must undergo, even less is known about the effects of processing on the active compounds. A research abstract presented at the 1999 Institute of Food Technologists (IFT) annual meeting indicated that cichoric acid, one of the active components of echinacea, was sensitive to processing treatment.[4] Thus, foods containing echinacea that require heating, such as soup, may lose efficacy. The same abstract indicated that active compounds in valerian root, valerenic acids, and eleutheroside E of Siberian ginseng (*Eleutherococcus senticosus*) were not affected by the processing methods that were studied. More research is needed on the stability of herbal active components. However, even if one were to determine that one compound was affected by processing, it would still need to be determined whether the whole herb in a food product becomes ineffective.

E. Safety Dilemma

Another key question for herbal additives to food is the safety of the herb itself. Examples of herbs with known dangers include chaparral, ephedra, blue cohosh, and yohimbe to name but a few. Other safety concerns include potential interactions with other medicines (e.g., anticoagulants and ginkgo biloba), and the possibility of contaminants, e.g., lead, in herbal preparations.

In many cases, the functional or active ingredients in an herb may have druglike qualities. The appropriateness of providing such an herb in a food product for general consumption needs to be considered. Allergies exist to some herbal preparations, so common allergens would need to be clearly identified in ingredient lists. Herbal preparations are by definition rather dilute. Adding the herb to a food may increase the dilution. What happens when one starts using the extracts? Are the active compounds now more concentrated and thus potentially stronger? How much is effective? How much is too much? Concerns are raised regarding unintentional consumption and consumption by children. These are some of the many unanswered questions surrounding functional foods with herbal ingredients.

IV. LABEL STATEMENTS AND CLAIMS

When considering herbals in food products, the regulations for label statements and claims are different from those for herbal preparations that are sold as dietary supplements. As with all ingredients in a food, herbal compounds must be identified in the ingredient list by their common and usual name; "St. John's wort" instead of "*Hypericum perforatum*," for example. For foods with added herbal ingredients, these ingredients may be also identified in label statements such as "contains kava kava" so long as such statements are truthful and not misleading. Comparison statements such as "contains 50% more kava kava than brand X" must be accompanied by indications of the amounts of kava kava in each brand per serving. Other label terminology such as "high in" or "good source of" is not applicable to herbal compounds because there are no Daily Values set for these ingredients as there are for essential nutrients.

One of the more controversial elements of DSHEA, and the resulting FDA regulations, relates to claims. Health claims are those FDA-approved statements that link a food or food component to reduced risk of a disease, e.g., calcium and osteoporosis, or fruits and vegetables and cardiovascular disease. To date, no health claims have been approved for herbal ingredients. Under current regulations, new health claims may be proposed at any time, but must be supported by an authoritative statement from a recognized government entity (e.g., USDA, NIH, NAS). Further, the mention of a disease on a food label or in an advertisement in the absence of an approved health claim results in the product being considered an unapproved drug by FDA. Only approved drugs may claim to diagnose, cure, mitigate, treat, or prevent a disease. However, dietary supplement and food manufacturers may use what have come to be known as structure/function claims to promote the positive aspects of their products.

Structure/function claims are statements of an effect of an herb on a body function or structure (e.g., "promotes urinary tract health"), or on general well-being (e.g., "gives energy and stamina"). Structure/function claims must be accompanied by the following disclaimer: "This statement has not been evaluated by the FDA. This product is not intended to diagnose, cure, mitigate, treat, or prevent a disease."

Structure/function claims have been used on food (e.g., "calcium builds strong bones"), but not extensively. As the regulations stand now, structure/function claims on foods must relate to the nutritive value of the food, and not to another ingredient or property. For example, capsules of cranberry juice sold as a dietary supplement can carry the claim "promotes urinary tract health" while bottles of cranberry juice (a conventional food) cannot because the beneficial effects of cranberry juice have not been linked to nutrient components. However, "nutritive value" is not defined in the regulations as pertaining solely to essential nutrient composition,

TABLE 24.1
Common Herbs and Alleged Function

Popular Name	Scientific Name	Active Components	Alleged Function	Main Body System
Ginkgo biloba	*Ginkgo biloba*	Ginkolides: flavonoid glycosides and diterpene lactones	Memory, cognition	Brain, circulatory system
St. John's wort	*Hypericum perforatum*	Hypericin, pseudohypericin, flavonoids, naphthodianthroms	Antidepressant	Brain
Kava kava	*Piper methysticum*	Kavalactones	Antianxiety, relaxation	Brain
Valerian	*Valeriana officalis*	Valepotriates, valerenic acid, sesquiterpenes	Sleep inducement	Brain
Hawthorn	*Crataegus oxycantha*	Flavonoids, procyanidins, triterpene saponins, cardiactive amines	Cardiac insufficiency	Circulatory system
Echinacea	*Echinacea purpurea* *E. pallida* *E. angustifolia*	Echinosides, caffeic and ferulic acids, glycoproteins and polysaccharides	Immunomodulator	Innate immunity
Peppermint oil	*Mentha × piperita*	Menthol	Irritable bowel disease	GI tract
Ginger	*Zingiber officinale*	Gingerols and gingerdiols, volatile oils	Antiemetic, nausea	GI tract
Licorice	*Glycyrrhiza glabra*	Glycyrrhetic acid, triterpene saponins, flavonoids, and isoflavonoids	Expectorant	Respiratory system
Saw palmetto	*Serenoa repens*	Not defined	Alpha-reductase inhibitor	Prostate gland
Cranberry	*Vaccinium macrocarpon*	Unknown	Bacteriostatic	Urinary tract
Feverfew	*Tancetum parthenuum*	Sesquiterpene lactones	Migraine prophylactic	Brain

and so further definition of structure/function claims on foods is needed to level the playing field with dietary supplements.

The remainder of this chapter discusses individual herbs, their purported actions, active compounds, and clinical evidence that supports efficacy, if available. They are organized by the physiological system that they are reported to influence and are summarized in Table 24.1. When known, food products containing these herbs are given, or, when examples do not exist, potential foods are suggested for the particular herb. Although the authors have reviewed and evaluated the current literature, it must be kept in mind that negative data are not available because they are usually not published.

V. ACTIONS OF HERBS AND EVIDENCE OF EFFICACY

A. Nervous System

Touted as a memory booster, **ginkgo biloba** has been showing up in a variety of food products, usually with some aspect of brain activity in the name; e.g., *Brain Broo*, *Think*, *BrainWash*, and *Wise Guy*. As a dietary supplement, ginkgo is the third best-selling herbal remedy in natural food stores in the United States, after echinacea and ginseng.[2] In Germany, ginkgo leaf extract was the most commonly prescribed single-ingredient phytomedicine in 1996.[2] Ginkgo is prescribed in

Europe for the treatment of cerebral disturbances and circulatory disorders.[2] Recent evidence suggests that ginkgo may be useful for people with Alzheimer's disease and other forms of dementia (discussed below).

As sold in dietary supplement form, ginkgo is an extract of the leaves of *Ginkgo biloba*. This tree is a native of China, but seems to grow well in a range of climates. City dwellers who may not know the name of the tree are probably quite familiar with the foul-smelling mature seeds it produces!

The active compounds in ginkgo are flavonoid glycosides and novel diterpene lactones, collectively known as ginkgolides. The ginkgolides are inhibitors of platelet-activating factor and thus have consequences on circulation, blood coagulation, and inflammation. The mechanism by which gingko has its effect is thought to be its ability to improve microvasculature insufficiency. An increase in blood flow to the brain may be the reason for the slowing of the mental decline in dementia. This also has ramifications related to cardiovascular health and may be efficacious in treating tinnitis and vertigo.

Clinical trials have focused on cerebral insufficiency and peripheral arterial occlusive disease, and most of the research on *G. biloba* has been done in Europe. Published reviews indicate that most studies show clinically significant improvement.[5,6] Studies of patients with intermittent claudication show significant increases in pain-free walking distance for those treated with *G. biloba* extract.[6]

A standardized extract of ginkgo, called EGb 761, has been used in many clinical trials, making comparisons between studies easier and more valuable. Indeed, many studies have shown the ability of EGb 761 to improve or slow the dementia related to Alzheimer's disease.[7–9] A meta-analysis to determine the effects of gingko on cognitive function in patients with Alzheimer's disease located 50 trials. The authors determined that the majority of the studies did not provide clear diagnoses of dementia and Alzheimer's disease. Of the four studies that met all of the inclusion criteria for the analysis, there was a small but significant effect of ginkgo on cognitive function in Alzheimer's disease.[10]

The idea that gingko slows the rate of dementia appears to have been extrapolated to suggest that consumption of this product will make us smarter, better able to concentrate, and improve our ability to focus. No research has been able to support this notion. Because of the antioxidant compounds in ginkgo, its role in functional foods may be beneficial. Gingko has been added to bars and beverages, including teas.

In clinical trials, mild side effects including headaches, gastrointestinal disturbances, and skin allergy have been noted (Table 24.2). Individuals who are already taking anticoagulant medication (e.g., warfarin or aspirin) or other dietary supplements with blood-thinning properties (e.g., vitamin E, garlic, ginger) should not take ginkgo without first consulting with their physician. Safety of ginkgo for pregnant and lactating women or for infants and children has not been established.[6] As with most herbs, no studies have been conducted on the effectiveness of *G. biloba* when consumed as a food product.

St. John's wort, *Hypericum perforatum*, is a widely prescribed antidepressant in Europe and has become a top-selling dietary supplement in the United States. Its common name (*wort* is Old English for *plant*) derives from the traditional belief that the plant blooms on the anniversary of the execution of St. John the Baptist. Although it has also been claimed to have antiviral and anticancer properties, it is the antidepressive effects of St. John's wort that have garnered the most attention for researchers and consumers.

Hypericum perforatum is a bushy plant with characteristic bright yellow flowers. It is the flowers and tops of the plant that are considered the active parts. A number of compounds may contribute to the activity of St. John's wort, including naphthodianthroms, flavonoids, xanthose, and bioflavonoids,[11] although hypericin and pseudohypericin are the compounds that have been the subject of most investigations.[5]

The exact mechanism of action of St. John's wort has not been well defined. *In vitro* evidence initally suggested monoamine oxidase (MAO) inhibition, but this is unlikely given the wide use in

TABLE 24.2
Adverse Effects, Potential Drug Interactions, and Contraindications of Common Herbs

	Adverse Effects	Potential Drug Interactions	Contraindications
Ginkgo biloba	Headaches, GI disturbances, skin allergy, one case of spontaneous hematomas	Augments anticoagulants such as warfarin, NSAIDs	No data on pregnancy and lactation
St. John's wort	Photosensitivity, dose-related serotonergic symptoms, dizziness, sedation	MAO Inhibitors, potentiates other CNS depressants	No data on pregnancy and lactation
Kava kava	Yellow discoloration of the skin, dose-related liver metabolic abnormalities	Alprazolam, potentiates other CNS depressants	Pregnancy and lactation, depression
Valerian root	Headaches	Barbiturates, potentiates other CNS depressants, perhaps alcohol	No data on pregnancy and lactation
Hawthorn leaves and berries	Dose related sedation, hypotension, arrhythmia	Additive with digoxin	Severe cardiac insufficiency No data on pregnancy and lactation
Echinacea	Allergy	Offsets corticosteroids, cyclosporin	Multiple sclerosis, tuberculosis, autoimmune diseases
Peppermint oil	None reported	None known	Pregnancy, gall stones, bile duct occlusion, severe liver damage
Ginger root	None reported	Augments anticoagulants	Gall stones, postsurgical nausea
Licorice	High blood pressure due to sodium, water retention, and potassium loss	Hypokalemia potentiates digoxin; diuretics, anthraquinone-containing laxatives; offsets corticosteroids, cyclosporin	Cardiac, liver, or renal failure; pregnancy
Saw palmetto berries	None reported	Other hormonal therapies	Pregnancy
Cranberry	None reported	None reported	None reported
Feverfew	None reported	Augments anticoagulants, NSAIDs may reduce effectiveness	Pregnancy and lactation

NSAIDs = nonsteroidal anti-inflammatory drugs; CNS = central nervous system; MAO = monoamine oxidase.

Europe without the reported side effects usually associated with MAO inhibitors (MAOI).[12] Other studies suggest effects on several neurotransmitters including gamma-aminobutyric acid (GABA) affinity, activation of dopamine receptors, and inhibition of serotonin receptors. It is possible that small additive effects on several neurotransmitters combine to give the antidepressant effect.

A large-scale clinical trial is currently under way in the United States comparing St. John's wort with fluoxetine hydrochloride (a common prescription medication for depression) and placebo in the treatment of mild to moderate depression. Existing data are encouraging. A recent meta-analysis compared outcomes of 23 randomized trials, determining a pooled estimate of the responder rate ratio (responder rate in treatment group/responder rate in control group). Linde et al.[13] found that hypericum extracts were superior to placebo, and equal to standard antidepressant drugs when standard depression scales were used. In a review of many of the same studies, Volz[14] came to the same conclusions regarding the efficacy of St. John's wort. However, both reports cite a variety of

shortcomings in the existing data including variations in subject population, extract preparation, length of interventions, and dosages.

In dietary supplement form, most preparations are standardized extracts (0.3% hypericin), although products may have widely varying contents. The dosage given in clinical trials is usually 300 mg three times per day. Side effects are minimal, but have included photosensitivity, gastrointestinal upset, dizziness, sedation, restlessness, and constipation.[5] St. John's wort should not be taken in combination with other antidepressants, and its safety in pregnant and lactating women and in children is unknown. Like most herbal supplements, long-term safety has not been adequately evaluated, although the wide use of St. John's wort in Europe has not suggested any specific concerns.

One of the major concerns about St. John's wort is not related to its efficacy or safety, but rather to the wisdom of individuals choosing to self-medicate with a dietary supplement instead of seeking professional help for depression.[15] Clinical depression is a serious condition that should be evaluated by a health professional prior to treatment.

As its fame grows, so does the appearance of St. John's wort in food products. Tea, juice blends and other drinks, soups, and snack foods with added St. John's wort suggest "calming," "relaxing" effects of these products. No studies are available to lend weight to these implied claims, however, since St. John's wort has not been tested for efficacy when consumed as part of a food.

Kava chips, kava chocolate, kava juice — all to relax and "mellow out" after a high-stress day. After all, traditional ceremonies in Hawaii and other locations in the South Pacific used kava beverages as natural intoxicants. In addition to its relaxing effects, kava supplements are also promoted for antianxiety effects. Animal studies have also found anticonvulsive, antispasmodic effects.[2]

Traditional kava beverages are prepared from the root of the *Piper methysticum* plant. Although the active ingredient(s) has not been definitively identified, a group of at least six kavalactones are thought to be responsible for the sedative and intoxicating effects. To date, the mechanism of action has not been defined. *In vitro* studies suggest that kavalactones affect benzodiazepine or GABA-binding sites, but results have been conflicting.

Very few controlled clinical trials have been conducted using kava. The kava extract used in clinical trials (WS 1490) is standardized to 70% kavalactones. Volz and Kieser[16] described a 25-week placebo-controlled trial of kava with outpatients who had one of several nonpsychotic anxiety disorders. Outcomes on anxiety scales and self-reported impressions showed improvement in the kava group. Like other clinical trials to date, conclusions from this study are limited due to the heterogeneity of the subject group, and the likelihood of depression as a comorbidity in a number of subjects.[5]

Adverse effects in clinical studies have been rare. Kava dermopathy (a temporary yellowish discoloration of the skin, hair, and nails) has been reported with higher doses, and heavy use has been suggested to cause liver-related metabolic abnormalities, including increased liver enzymes.[17] Because of its effects on the central nervous system, kava should not be used in conjunction with alcohol, barbituates, or psychopharmacological agents. Long-term safety is unknown.

Valerian root (species: *Valeriana officalis* L.) is well known in many cultures as a natural sleep aid. It is also promoted as having anxiolytic and antispasmodic activity. In Germany, it is approved as a mild sedative and sleep aid.[2] Animal studies support the benefits of valerian as a mild hypnotic agent, but are inconsistent for effects relative to anticonvulsant or antidepressant activity. Several small human clinical trials have found valerian to be efficacious as a mild sedative, showing a significant decrease in sleep latency as compared to placebo.[5,11] A larger randomized, placebo-controlled trial (n = 128) found that 400 mg of aqueous valerian extract before bedtime improved sleep quality and reduced sleep latency with no residual sedation upon awakening.[18]

The active ingredients in valerian are likely valepotriates and sesquiterpenes.[17] Both compounds are known to have sedative effects, and valepotriates are known to be cytotoxic. However, therapeutic preparations do not contain valepotriates as these are thermolabile and chemically unstable.

Valerian has also been shown to be high in GABA.[5] The mechanism of action, however, is unknown. Extracts of valerian have affinity for GABA receptors, possibly due to the high GABA content, but this may have no sedating effect since GABA does not readily cross the blood–brain barrier. Other postulated mechanisms involve 5-hydroxy tryptophan and adenosine receptors.

Reported dosages in the literature vary widely; from 500 mg to 12 g per day, given in single bedtime or divided doses.[17] A single case of overdose has been reported, but, otherwise, few adverse effects have been noted. There is no evidence that valerian is addictive. However, like most herbal remedies, there is insufficient evidence of safety in pregnant and lactating women. In addition, most sources recommend avoidance of alcohol and other CNS depressants when taking valerian.[5,11,12]

Valerian has been approved for use as a flavoring for foods and beverages, and is considered GRAS. Few examples of products containing valerian have been found other than teas and other beverages touted for relaxation. No studies have been done on valerian as a food ingredient.

B. Heart and Circulation

The **hawthorn plant** *Crataegus oxycantha* has been traditionally used for a number of cardiac complaints such as angina, hypertension, arrhythmia, and congestive heart failure. The medicinal parts of the hawthorn are the leaves, flowers, and berries, used singly or in combination. There is sufficient evidence in the literature for the Commission E to classify the hawthorn leaf with flower combination as an approved herb for the treatment of mild cardiac insufficiency. Studies show that 160 to 900 mg/day of an aqueous-alcoholic extract was effective for a period of time that lasted up to 56 days; however, no pharmacokinetics of the drug in humans are available. An improvement in subjective findings, as well as an increase in work tolerance, ejection fraction, and a decrease in pressure/heart rate product, was indicated. Animal studies in rats show that *in vitro* pretreatment of the isolated heart with dried hawthorn extract was protective against arrhythmia[19] and cell membrane damage[20] caused by no-flow ischemia. A tincture of hawthorn berries was effective in preventing lipid deposits in the liver and heart,[21] increasing low-density lipoprotein (LDL)-receptor activity and reducing cholesterol synthesis[22] in rats fed an atherogenic diet.

The active compounds of the hawthorn are the flavones, flavanols, and oligomeric procyanidins (catechins and epicatechins). Other potentially active compounds are the triterpene saponins, phenolics, and a few cardioactive amines. Like many of the other botanicals, the antioxidant and antithrombotic activities are likely to be responsible for improvement in cardiovascular symptoms. The historical claims for improving blood circulation, decreasing blood pressure, and reducing varicose veins have not been substantiated in either human or animal studies.

Hawthorn has been added to beverages; for example, Hansen's Naturals makes a juice called Intellijuice that contains ginkgo and hawthorn. Tribal Tonics makes Relaxation Cocktail containing hawthorn berry, kava, and chamomile. Neither of these beverages suggests management of vasculature health. A precedent has been established, however, although not with an herbal preparation. The HeartBar™ formulated by Cooke Pharmaceutical is the first nonprescription medical food on the market for dietary management of vascular disease. The active component of this bar is L-arginine, a precursor to nitric oxide. A functional food containing hawthorn has potential to be marketed for cardiovascular health as well. More controlled, randomized, clinical trials with humans are needed before this herb is found to be efficacious or accepted as a medical food.

C. Immune System

Herbs that are designed to stimulate the immune system include echinacea, astragalus, cat's claw, goldenseal, and pau d'arco (also known as lapacho). Each herb appears to influence different branches of the immune system. Echinacea is probably the best studied of the group.

The **echinacea plant**, also known as the purple coneflower, has been used for centuries. Although this is not an argument for efficacy, it does indicate some degree of safety. The herb is best known for its immunostimulating capabilities and is used for treating the common cold, flu, coughs, and bronchitis.[2]

Medicinally, three species of echinacea are important: *E. purpurea*, *E. angustifolia*, and *E. pallida*. Each has been alleged to have different medicinal properties, but little research has been done to compare them. The roots, the leaves, or the whole plant may be used in the dietary supplement preparation. Standardized extracts may be aqueous extracts or ethanol extracts. The active components of echinacea are flavonoids, immunostimulating glycoproteins and polysaccharides, and caffeic acid and its derivatives.

Five randomized, placebo-controlled studies involving a total of 134 subjects have been reported.[23] Two of the five studies showed an increase in phagocytic activity of peripheral blood neutrophils. Each of the five studies was done with different amounts of echinacea from different plant parts and in different preparation combinations. The inconsistent results underscore the importance of knowing the species of echinacea, the method of extraction, and the route of administration. A larger study by the same principal author looked at the ability of echinacea to prevent upper respiratory infection (URI).[24] "Time to event" was the primary outcome measure on 302 subjects who were healthy at the start of the study. Oral ingestion of the herb preparations or a placebo for 12 weeks did not prevent URI. Grimm and Muller[25] completed a study with 109 healthy subjects using fresh juice of *Echinacea purpurea* or placebo juice. The number of subjects having a URI, the number of URI per subject, and the duration of any colds was measured. There was no significant difference in these measures between the echinacea group and the placebo.[25]

Studies that examine the prophylactic ability of echinacea to prevent illness show little to no significant benefit. This is likely because of its mechanism of action, an enhancement of phagocytic cell activity. Echinacea would not be expected to prevent colds or flu; rather, it would be expected to enhance killing of the invading organisms, thus is more likely to be a therapeutic herb rather than a prophylactic herb. Recommendations for echinacea use suggest that it be consumed at the onset of symptoms. Because of the evidence that echinacea affects the phagocytes, long-term ingestion of echinacea may potentially do more harm than good. Increased reactivity of the phagocytic system may result in generation of more free radicals. Free radicals, in turn, may then cause damage to the host. Many publications caution not to take echinacea for more than 6 to 8 weeks.

Other studies have been done on echinacea, but are published in German. Fortunately, Barrett et al.[26] recently reviewed seven German studies published between 1984 and 1997. All studies were classified as double-blind, randomized, clinical trials with a total of 910 subjects. The outcome measure for two studies was duration and severity of flulike symptoms and the outcome measure for five studies was duration and severity of URI symptoms. Outcome was determined by self-reporting as well as by physical examination. All seven studies showed a significant benefit due to echinacea. Barrett notes that the quality of these published trials is moderate, but, nonetheless, it appears that the use of echinacea to modify the severity and duration of cold symptoms is supported.

In the United States, echinacea was the top-selling product in natural food stores in 1997.[2] Because of its popularity, several functional foods have been designed with echinacea. Echinacea can be found in snack foods, beverages, and soups. More studies need to be done on the shelf life and heat stability of the active compounds.

D. Digestive System

Almost every herb listed in general reference books seems to have some influence on the digestive system. The alleged use of the botanical is to cleanse or purify the body. Cleansers and tonics are very difficult to study. Some of the herbs found to stimulate digestive juices include wormwood,

dandelion, horseradish, chicory, and tarragon. Precautions should be noted for wormwood (daisy family) as this genus may contain thujone, a small molecule that has been responsible for convulsions, hallucinations, and kidney failure. Fennel is supposed to act as a carminative, a substance to relieve gas, cramps, and bloating. Aloe, when taken internally acts as a laxative. Peppermint oil has been used in the relief of irritable bowel syndrome (IBS) and gastritis.

A meta-analysis of clinical trials done with **peppermint oil** (*Mentha* × *piperita*) described eight studies in which there was a global improvement in the symptoms associated with IBS. The main active component in the oil is menthol. The authors of the meta-analysis noted several problems with existing studies, especially with the diagnosis of IBS. Only one out of eight trials properly diagnosed the condition. Three of the eight studies did not state their inclusion criteria. So although the global outcome suggested that peppermint oil is effective, the quality of the studies makes the data suspect. The authors of this meta-analysis note that in early studies, peppermint oil acted as a calcium antagonist in human and animal studies. The consequence of calcium antagonism in the gut is relaxation of gastrointestinal smooth muscle, thus possibly promoting bowel rest. The amount of peppermint oil needed to affect the gut is likely far too much to be palatable in a food.

Another herb that has been suggested to promote gastrointestinal well-being is **ginger** (*Zingiber officinale*). Ginger is a common culinary spice; however, levels that are recommended therapeutically (2 to 4 g of the rhizome daily) are far greater than what is incorporated into a food as a seasoning. Clinical trials with ginger have focused on preventing postoperative nausea and motion sickness. One study found that ginger worked to prevent postoperative nausea as effectively as metoclopramide and better than placebo.[27] A ginger extract (200 mg) increased stomach motility,[28] but 1 g of the whole root did not alter gastric emptying.[29] Also, 1 g of the root did not prevent experimentally induced motion sickness,[30] but in another study, 1 g of the root reduced the tendency to vomit on the high seas.[31] Available data are inconclusive on the antinausea mechanism of ginger, but it is generally believed to act differently from Dramamine®. Benefits, if research bears it out, would be obtained with gram quantities of the root and therefore may not be palatable for a functional food. Teas, however, are currently in use as a mode of administration, suggesting potential for ginger to be used in a beverage to prevent nausea.

E. Respiratory System

The coughs that accompany illness are sometimes treated with over-the-counter medicines designed to act as expectorants. The **licorice root** (*Glycyrrhiza glabra*) is claimed to act as an expectorant although according to the *PDR of Herbal Medicines*[32] this has only been researched in rabbits. Licorice root has also been shown to cause a loss of body potassium; however, the mechanism by which it does this is unclear. The importance of replacing lost potassium should be stressed and perhaps potassium could become a component of a functional food containing licorice root.

F. Urinary System

There has been substantial interest lately in the benefits of **cranberry** (*Vaccinium macrocarpon*) for urinary tract health.[33] Cranberry appears to act as an antiseptic while at the same time preventing bacterial adherence to the epithelial cells of the urinary tract.[34] Other berries of the same family such as the blueberry (*V. angustifolium*) and bilberry (*V. myrtillus*) may also aid in urinary tract health. Bearberry (*Arctostaphylos Uva-Ursi*), a different family, is also indicated in urinary tract infections. The latter three have not been well studied in this respect at all.

Saw palmetto (*Serenoa repens*) is a very common palm plant indigenous to the southeastern United States. The saw palmetto berry is used in the treatment of benign prostatic hyperplasia (BPH); 40% of men over the age of 70 have this condition. Frequent urination, nocturia, weak stream, incomplete emptying, and hesitancy characterize BPH. The enlargement of the prostate is

thought to occur via the action of dihydrotestosterone (DHT). DHT is derived from testosterone via an alpha reductase enzyme. The action of DHT is anabolic, hence the growth of the prostate. Treatment for this condition uses drugs designed to inhibit the alpha reductase enzyme. Saw palmetto berries contain compounds that inhibit the alpha reductase enzyme.

A meta-analysis of 18 randomized trials of the efficacy of saw palmetto berries for BPH symptoms was published in 1998.[35] Of a total of 2939 men that took part in these studies, 1118 were in studies that compared saw palmetto to placebo and 1821 compared saw palmetto to finasteride, a drug currently used in BPH treatment that acts as an alpha reductase inhibitor. The self-reported improvement from the men favored saw palmetto berries over placebo. Similarly, relief from nocturia and an increase in peak flow rates favored saw palmetto berries over placebo. When finasteride was compared with saw palmetto berries using the International Prostrate Symptoms Scale, no difference was observed between finasteride and saw palmetto, meaning that the saw palmetto was as effective as the drug. The authors noted two advantages of the berry over the drug: (1) saw palmetto berry is less expensive than finasteride and (2) erectile dysfunction was noted in 5% of the men on the drug, whereas there were no such side effects in the saw palmetto group.

Shortcomings of this meta-analysis included inconsistent reporting of outcomes, no attempts to assess the quality of blinding, only three trials used results from standardized, validated urological symptom scales, and the different doses and preparations of saw palmetto used in each study.

Other botanicals that are becoming better known for the ability to reduce the symptoms associated with BPH include *Pygeum africanum* and *Urtica dioica*.[36,37] Less is known about these herbs; however, *Pygeum* acts as an alpha reductase inhibitor.

For mild BPH, saw palmetto berries may be beneficial. Their actual benefit in a functional food, rather than in capsule form, is questionable, however. A quote from the 1696 diary of a young Quaker merchant, Jonathon Dickinson, said, "We tasted them but not one amongst us could suffer them to stay in our mouths; for we could compare the taste of them to nothing else, but rotten cheese steeped in tobacco."[38]

G. Musculoskeletal System

There are a number of herbal preparations designed to act as analgesics (relieve joint and muscle pain) and anti-inflammatory agents to reduce swelling and pain. The bark of the white willow is known to contain salicylates, which is the compound that is aspirin. **Feverfew** (*Tancetum parthenuum*) is an herb resembling the daisy that has been reported as a prophylactic treatment for migraine headaches.[39,40] The active components of feverfew are thought to be the sesquiterpene lactones.

In 1985, subjects that were already taking feverfew preparations were studied.[40] Half of the group was given a placebo, while the other half was given standardized capsules of feverfew. There was a significant increase in frequency and severity of migraine among those on the placebo, while those that took the feverfew-containing capsules had no change in their attacks. In a crossover design study, patients who took feverfew had a reduced number and severity of attacks compared with the placebo group.[39] An *in vitro* study suggested that the mechanism by which feverfew worked was by inhibition of 5-lipoxygenase and cyclo-oxygenase enzyme activities.[41] The five clinical trials that have been published suggest a beneficial effect of feverfew compared with placebo. However, the authors of a meta-analysis advise that the efficacy of feverfew has not been established beyond a reasonable doubt.[42]

VI. SUMMARY AND CONCLUSION

Adding botanicals to foods to create functional products raises many issues including appropriate regulation, safety, stability, and efficacy. Individual food–herbal dynamics will create even more

challenges, including ways to mask the unpleasant flavors of some herbs. Many gaps exist in our current knowledge such that caution on the part of both the manufacturer and consumer is in order. However, as our knowledge base grows and confirms the suspected actions of many herbal compounds, our future food supply could be dramatically different from what it is today.

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25 Probiotics and Prebiotics

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I. PROBIOTICS

Within the definition of functional foods there is a subset of foods believed to be good for health that are produced by or that contain live microorganisms. These foods are called probiotics. The earliest definition of a probiotic was very narrow and applied specifically to animals (see Table 25.1). As (1) the metabolism of bacteria became better defined, (2) more studies pointed out the role that microorganisms play in human health and disease resistance, and (3) more microorganisms were identified that may be included in probiotic products, there has been a need to expand the definition of a probiotic. It should be noted that discussions of probiotics are usually limited to bacteria; the use and importance of yeasts in probiotic foods have not received much attention.

A. Criteria for Probiotics

As researchers and food manufacturers started to appreciate the potential for including microorganisms in probiotic foods, various criteria were suggested to screen candidate microorganisms that would at the same time satisfy regulatory bodies and ensure consumer acceptance (see Table 25.2). It is difficult to rank the criteria in order of importance; the first three criteria address practical

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TABLE 25.1
Evolving Definitions of Probiotics

"Animal feed supplements that have a beneficial effect on the host animal by affecting its gut microflora"[1]

"A live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance"[2]

"A viable mono- or mixed culture of micro-organisms which, when applied to animals or man, beneficially affects the host by improving the properties of the indigenous microbiota"[3]

"A microbial preparation which contains live and/or dead cells including their metabolites which is intended to improve the microbial or enzymatic balance at mucosal surfaces or to stimulate immune mechanisms"[4]

aspects of selection of appropriate microorganisms for inclusion in probiotic foods, and the later two may be more important from a consumer acceptance and regulatory point of view. The ideal probiotic bacteria would possess all of the criteria. However, at this time, the list of bacteria that meet several of the criteria and, in particular, those that have sound scientific evidence of efficacy and that have been incorporated into foods, is not long.

Several of the criteria listed in Table 25.2 are obviously related. To be effective, the probiotic bacteria must arrive at the site of action in large enough numbers to exert their effect. This may most easily be achieved by using resistant bacteria or by providing large numbers of live bacteria when consumed, to compensate for losses during passage through the upper gastrointestinal (GI) tract. Knowledgeable consumers are now looking for products that advertise that they contain live bacteria. Meanwhile, manufacturers are becoming more aware of the need to provide accurate information about the levels and types of bacteria in their products.[4] The setting of a minimum number of viable microorganisms in a food to ensure probiotic effects is an important decision. The Fermented Milks and Lactic Acid Bacteria Beverages Association in Japan has set a minimum of 10^7 bifidobacteria/g or ml.[10] Others have suggested a lower level (10^5).[5] Recent results would indicate that metabolic changes can be observed at such levels of microbial intake.[11] However, experimental evidence in which various levels of bacteria were fed and viable numbers counted after passage through the stomach would indicate that probiotic products may have to deliver even higher ($>10^{10}$ to 10^{11}) levels of microorganisms to survive the conditions of the GI tract.[12] This conclusion is supported by *in vitro* tests done on a variety of potential probiotic bacteria using simulated digestive systems.[13,14] This wide diversity of opinion may reflect only the differences in the ability of different bacteria to survive passage through the stomach. To set one level for all bacteria may be unrealistic, because the numbers of bacteria required to produce a certain metabolic change will vary depending on the effect to be achieved.

TABLE 25.2
Proposed Criteria for Microorganisms to be Included in Probiotic Foods/Drinks[5–9]

1. Microorganism of human origin
2. Resistance to acid conditions of stomach, bile, and digestive enzymes normally found in the human GI tract
3. Ability to colonize human intestine
4. Safe for human consumption
5. Scientifically proven efficacy

Only bacteria that can survive passage through the stomach and can colonize the intestines will exert any effect on the host. This was most clearly shown by Pedrosa and colleagues.[15] In healthy elderly persons fed yogurt containing *Lactobacillus bulgaricus* and *Streptococcus thermophilus*, these two organisms could not be found in intestinal contents after feeding and there were no changes in three bacterial enzyme activities. When healthy subjects were fed live *Lb. gasseri* (*Lb. acidophilus* strain MS 02), a human strain capable of adhering to intestinal cells, the organism was found in all subjects and the activities of β-glucuronidase, nitroreductase, and azoreductase were all significantly reduced. This study emphasizes the need to measure both the fate of the ingested bacteria and metabolic effects to ensure proper interpretation of results. This is particularly true when no metabolic response is observed.

Various reports have included lists of bacteria that can be considered as candidates for inclusion in probiotic products (Table 25.3). The whole premise of a functional food is that certain commonly eaten foods contain active ingredients that can fight or prevent disease and infection. With advances in analytical techniques the identification of new active ingredients is occurring every day. For probiotics, however, the identity of the active ingredient may not be as straightforward, because there is always more than one possibility. The active agent could be one or more of the live microorganisms in the food, or it could be a metabolite produced by one of the microorganisms, or it could be a fermentation product that has been formed due to the action of the microorganism(s) on the original product. Indeed, depending on the probiotic in question each of the above arguments had been used to explain the beneficial effects of the probiotic.

B. Probiotic Products on the Market

In Japan there is a long tradition of believing that health is dependent on food and in particular that the maintenance of a population of beneficial intestinal bacteria is important. The Japanese in 1981 established the Japan Bifidus Foundation to encourage research on bifidobacteria which in part may explain why, by 1993, 53 probiotic products containing bifidobacteria were for sale in Japan.[10] Japan is often held up as a example of how cooperation between industry and government health agencies can lead to system of approval and labeling of foods that are good for health. The "foods for specific health uses" or FOSHU system provides consumers with products with a distinctive logo indicating that the product (or product ingredients) have been judged as being good for health. There are 11 categories of functional components that qualify under the FOSHU system. Three of these categories, namely, dietary fiber, oligosaccharides, and lactic acid bacteria, refer specifically to intestinal function and control. By 1998, over 70% of products being marketed with the FOSHU label were directed toward intestinal bacteria and GI conditions.[16]

Reuter [4] pointed out that in both Germany and Switzerland there is wide consumer interest in probiotic products. He lists 21 products (produced in seven European countries) for sale in Germany of either a yogurt-type or a yogurt drink format in 1997. Regulatory concerns may have slowed the introduction of such products in North America.

II. PREBIOTICS

The concept of a prebiotic arose from two observations: (1) bacteria like any other living organism have (sometimes specific) nutrient requirements[17] and (2) some nutrients, particularly complex carbohydrates, pass undigested into the colon where they are utilized by resident bacteria.[18] In 1995 Gibson and Roberfroid[19] defined a prebiotic as a nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and /or activity of a limited number of bacteria in the colon. Rather than supplying an exogenous source of beneficial bacteria, the concept of a prebiotic proposes to increase the number of certain target bacteria already in the colon. The targeting of *in situ* bacteria obviously satisfies most of the criteria listed in Table 25.2. Attention has centered mainly on the increase of *Lactobacillus* and *Bifidobacterium* as the two main groups

TABLE 25.3
Possible Probiotic Microorganisms [5,7,8]

***Lactobacillus* bacteria**
Lb. acidophilus
Lb. acidophilus LC1
Lb. acidophilus NCFB 1748
Lb. plantarum
Lb. casei
Lb. casei Shirota
Lb. rhamnousus (strain GG)
Lb. brevis
Lb. delbrueckii subsp. *bulgaricus*
Lb. fermentum
Lb. helveticus
***Bifidobacterium* bacteria**
B. bifidum
B. longum
B. infantis
B. breve
B. adolescentis
Other bacteria
Streptococcus salvarius subsp. *thermophilus*
Lactococcus lactis subsp. *lactis*
L. lactis subsp. *cremoris*
Enterococcus faecium
Leuconostoc mesenteroides subsp. *dextranicum*
Propionibacterium freudenreichii
Pediococcus acidilactici
Escherichia coli
Yeasts
Saccharomyces boulardii

Note: The identification and classification of bacteria and yeasts change as more sophisticated methods are used. The nomenclature used throughout this chapter is that found in the references cited to avoid confusion.

of "friendly bacteria" to increase.[20–23] Variation in individual response is often high and as cautioned by Roberfroid[24] the prebiotic effect may depend on the initial level of target bacteria. The carbohydrates of interest as prebiotics are listed in Table 25.4.

The goal of increasing selected bacteria by feeding prebiotics often has been the production of various short-chain fatty acids (SCFAs) because of their impact on the lower gut environment, metabolism, and disease prevention.[25–27] The SCFAs are quickly absorbed and can serve as an energy source for the host, especially between meals. They contribute to fecal pH and thereby influence colonic function and perhaps the risk of cancer.[28] Other beneficial health effects have been proposed. However, as in the case of the effects of feeding a prebiotic such as galactooligosaccharide on constipation, the results are not conclusive.[29]

The combination of live bacteria in a food and the inclusion of nutrients (usually sugars) that can be used by those bacteria as the two traverse the GI tract has resulted in what has been termed

TABLE 25.4
Some Proposed Prebiotics[31,32]

Neosugars
(GFn n = 1 – 4
Inulin-like fructans (fructo-oligosaccharides of chain length up to 60 units)
Soy oligosaccharides
Galacto-oligosaccharides
Isomalto-oligosaccharides
Gentio-oligosaccharides
Xylo-oligosaccharides
Lactulose (fructose-galactose disaccharide)
Raffinose
Stachyose
Sorbitol
Xylitol
Palatinose
Lactosucrose

symbiotic foods.[24] The most popular combination to date appears to be *Bifidobacterium* and fructooligosaccharides, but other combinations are possible, if not practical. [23,30]

III. MICROBIOLOGY OF THE GASTROINTESTINAL TRACT

The human microflora is complex, difficult to study, and is influenced by many factors. Study of the microorganisms that populate human GI tracts is hampered by the fact that sampling opportunities are limited, variation between individuals is great, there is no totally relevant animal model, such research is time-consuming and expensive, and advances in this area of research are obviously dependent on advances in microbiology. Even the simplest questions, such as how many and what kind of bacteria are important in the human GI tract, are not easy to answer or to find a consensus in the literature. Indeed, at least one author has stated that due to difficulties in obtaining proper samples, the crudeness of the methods used to count the various types of bacteria, and the short duration of most diet intervention studies, very little is really known about the human microflora and factors that affect it.[33] To date, most of our understanding of the human microflora comes from analyses of fecal contents, which may severely limit our understanding of events farther up the GI tract.

There is agreement about the changes (in general terms) in the bacterial population that occur during the passage from newborn to infant to adult. Vaginally delivered babies have nearly sterile GI tracts, which soon become colonized, either with large numbers of *Bifidobacterium* and *Lactobacillus* in the case of breast-fed babies or *Bacteroides* sp. and *Eschericha coli* in the case of bottle-fed babies.[34] *Bifidobacterium* are considered as desirable, and so attempts have been made to add prebiotics to infant formula to encourage the growth of Bifidobacteria.[35] By adulthood, over 400 different species may be present (see Table 25.5), but only the most abundant bacteria have been well identified. Resident bacteria can be found starting with the mouth and working on down the GI tract. The stomach and the upper small intestine may have as many as 10^5 colony-forming units (CFU) the ileum 10^7, and the colon 10^{11} to 10^{12}.[36] Anaerobic bacteria outnumber aerobic by a factor of 1000 to 1.[37]

The bacteria that reside in the human intestinal tract have both beneficial and harmful effects on the host. The production of vitamins, SCFAs, some proteins, the role played in digestion and absorption of nutrients, the production of protective bacteriocins, and the stimulation of the immune system are all positive effects of the intestinal microflora.[10,31,38] At the same time, intestinal bacteria

TABLE 25.5
Bacteria Found in Human Intestinal/Fecal Samples[39,40]

Bacteroidaceae	Eubacteria
Peptococcaceae	Bifidobacteria
Streptococci	Enterobacteriaceae
Lactobacilli	Vellonella
Clostridium perfringens	Clostridia
Magasphaera	Fusobacteria

produce carcinogens and mutagens directly during their metabolism, and also enzymes that convert digesta contents into carcinogens and mutagens. Products of fermentation such as ammonia, amines, and phenols may be harmful and some bacteria are pathogenic.[39,40] Mitsuoka[39] and Gorbach[40] list the many enzymatic reactions of intestinal bacteria and the impact on health, disease, and infection.

Because of the difficulties in identifying and enumerating various microorganisms that inhabit the GI tract, several authors have suggested that a more sensitive and perhaps more relevant approach is to measure effects on the metabolic activities of the bacterial flora, because of their potential impact on disease.[9, 33,37] Carman and colleagues[41] used the term *microflora-associated characteristics* (MACs) and argued that changes in MACs brought about by dietary (probiotic or not) interventions were true indicators of changes in the intestinal flora, and a more useful way of establishing the consequences of such changes. Goldin and Gorbach[42] used much the same reasoning when they measured bacterial enzymes associated with the production of carcinogens in the intestine. Table 25.6 lists the MACs suggested by Carman and colleagues.[41] Other characteristics could be added that also reflect changes to the intestinal tract.

IV. PROBIOTIC PRODUCTS

A. Yogurt

Yogurt is defined as a fermented milk obtained by specific lactic acid fermentation, brought about by *Lb. bulgaricus* and *S. thermophilus*. Other bacteria may be added to enhance organoleptic properties, or more recently, to increase the probiotic properties. Yogurt can now be found that contains *Lactobacillus*, *Streptococcus*, *Leuconostoc*, and *Bifidobacterium* bacteria. Pakistani yogurt or dahi has been shown to have an even more complex microflora.[43]

The amount of research on yogurt far surpasses any other probiotic product. Yogurt has been studied to determine its effects on lactase deficiency, cholesterol metabolism, immunity, infantile diarrhea, and certain cancers with varying levels of success.

TABLE 25.6
Microflora Associated Characteristics Indicative of Intestinal Microflora Changes

1. Cellular fatty acid profiles of washed bacteria pellet of fecal or cecal contents
2. Primary to secondary bile acid ratio
3. Ratio of primary to secondary sterols
4. Molar ratio of SCFAs in intestinal material

Source: Carman, R.J. et al., *Vet. Hum. Toxicol.*, 35 (Suppl. 1): 11–14; 1993. With permission.

1. Lactase Deficiency

Yogurt has been shown to be a diary product that may be tolerated by people with a lactase (β-galactosidase) deficiency. It is more accurate to refer to a lactase deficiency as opposed to a lactose intolerance. Lactase is the digestive enzyme found in the intestinal brush border responsible for the hydrolysis of milk sugar lactose into glucose and galactose. Lactase deficiency results in the buildup of unabsorbed lactose which acts osmotically to retain water. The deficiency is characterized by diarrhea, excessive flatulence, bloating, and abdominal pain after the ingestion of milk or milk products. Measurement of the enzyme activity, the concentration of lactose/glucose/galactose in digesta, or the measurement of breath hydrogen are ways of evaluating the treatment effects on lactase activity.

In 1984, two groups, Kolars and colleagues[44] and Savaiano and colleagues,[45] demonstrated that yogurt reduced the symptoms of lactase deficiency and they speculated this was due to the live bacteria in yogurt. Yogurt was said to be able to autodigest lactose and it was apparent that the enzyme responsible survived passage through the stomach.[46] Subsequent studies have built on this initial work and have added to the understanding of the mechanism involved. Yogurt, but not heat-treated yogurt, improves lactose digestion indicating that the live bacteria in yogurt are responsible, and long-term ingestion (8 days) does not seem to change this.[47,48] The bacteria in yogurt survive passage through the stomach due to the enhanced protective (buffering) properties of the yogurt compared with milk. However, the buffering capacity of yogurt may also slow hydrolysis until the digest passes to a point in the intestinal tract where the pH favors β-galactosidase activity.[47] The β-galactosidase activity in commercial yogurts has been found to vary depending on the manufacturer, whether fruit is added and whether the yogurt is frozen or not. Frozen yogurt that is pasteurized before freezing has no β-galactosidase activity. To overcome this, some manufacturers add starter culture to the pasteurized yogurt before freezing, but this does not necessarily increase enzyme activity.[49]

In addition to the *in vivo* bacterial hydrolysis there also appears to be a slower rate of stomach emptying after a yogurt load compared to milk. This effect may contribute to the enhanced digestion of lactose in yogurt.[50]

The beneficial effects of yogurt on healthy individuals may not be as obvious as for those who have a defined medical problem. Guerin-Danan et al.[51] saw few changes in the fecal bacteria, and the bacterial enzyme activity of healthy infants (10 to 18 months old) over a 1-month supplementation trial. Branch-chain and long-chain fatty acid levels were significantly reduced during yogurt consumption, however.

2. Cholesterol Metabolism

The research into the effects of yogurt consumption on blood cholesterol has been difficult to understand because of the contradictory results that have been reported over the years. It is now clear that in many cases, results and conclusions from one experiment cannot be compared with others because of differences in experimental protocol. The sex, age, general health status, initial cholesterol level, and level of activity all influence cholesterol metabolism. The diet before and during the experiment can also influence results, as can the time of day the yogurt is consumed, whether it is consumed with other food or not, and the position in the meal (beginning or end of meal). One of the most important details of many yogurt–blood cholesterol experiments, namely, the type of yogurt fed (including details of the levels and types of bacteria in the test yogurt or fermented milk), are often not described. This, together with the fact that proper controls for such feeding trials were often not included, reduces the scientific validity of many studies.[52]

The observation that the Maasai of Africa had low blood cholesterol levels (compared with Western standards) and were free of signs of coronary heart disease prompted Mann and Spoerry[53] to carry out their much reported study involving fermented milk. Yogurt trials carried out since that time use this as a starting point in spite of the fact that Mann[54] later emphasized that he believed

that it was a milk factor responsible for the hypocholesterolmic effect, which was enhanced by fermentation to yogurt. Taylor and Williams[52] list 12 publications (13 trials) in which yogurt has been fed in an attempt to lower blood cholesterol. As they point out, many of the study protocols can be criticized — too few subjects, too short of a feeding trial, no or improper control diets, and unrealistic feeding levels. Of the 13 trials, 8 reported reduced blood total cholesterol levels, 1 reported an increase, and 4 reported no difference from control. A recent study by de Roos and colleagues[55] can be added to the "no difference from control" group.

Various suggestions have been made regarding the possible active ingredient(s) or the mode of action of yogurt, which might affect cholesterol metabolism.[56] *In vivo* cholesterol synthesis may be related to and controlled by the availability of certain SCFAs in the gut; interest has centered on acetate and propionate.[57,58] Several bacteria have been shown to be capable of hydrolyzing bile acids, which would prevent reabsorption in the intestine and facilitate the elimination from the body. Bile acids are formed from cholesterol in the liver and therefore any increase in elimination of bile acids from the body would increase the rate of conversion of cholesterol to bile acids.

3. Immune Function

The organs of the immune system are varied and within this system the leukocytes or white blood cells provide specific or nonspecific immunity. A wide variety of parameters including levels of specific immunoglobulins, numbers of different cell types, and measurement of specific metabolite concentrations are used to measure immune function. At the present time several hypotheses have been put forward regarding how yogurt might improve the immune system. Interaction of the yogurt bacteria with intestinal bacteria could produce indirect effects or the gut associated or systemic immune system might be affected by metabolites of the bacteria themselves or fermentation products in the yogurt.[59]

Perdigon's group[60,61] has published several studies using animals that show that yogurt does improve immune function. Wheeler and colleagues[59] measured a wide variety of cellular, humoral, phagocytic, and mucosal immunity parameters, in patients with preexisting atopic disease and found no significant differences in any parameters whether the patients were consuming yogurt or milk (control). Spanhaak and colleagues[62] similarly reported no changes in immunity parameters in healthy subjects who had been fed milk fermented with *Lb. casei* strain Shirota even though fecal bacteria patterns were changed and fecal enzyme activities were significantly decreased.

Gill's[63] review of the literature emphasized the difficulties in comparing the results from different studies, but concluded that there is enough evidence to suggest that certain lactic acid bacteria, given at adequate levels of intake, can influence immune function.

4. Diarrhea

Diarrhea, particularly in young children, can be problematic because of the need to rehydrate the patient as quickly as possible, combined with the problems associated with decreased nutrient intake. The detrimental consequences of complete withdrawal of food from infants as a treatment have been defined, but withholding food during early stages of diarrhea is still widespread.[64] Fermented milk or yogurt can supply liquid and at the same time may supply natural antibiotics produced by the lactic acid bacteria to prevent or reduce the severity of infantile diarrhea. Gonzalez and colleagues[65] showed that milk fermented with *Lb. casei* and *Lb. acidophilus* could be used to prevent the incidence of diarrhea (17% vs. 59% for controls receiving unfermented milk) in infants 5 to 29 months old, and that the protective effect of the fermented milk appeared to be correlated to the level of fecal lactic acid bacteria. Isolauri and co-workers[66] used milk fermented with the human strain *Lb. casei* sp. strain GG to reduce the duration of acute diarrhea in children. This same strain of bacteria was shown to be effective in the prevention of antibiotic (erythromycin) associated diarrhea, due in part to its ability to colonize the intestinal tract.[67] However, these positive results appear only to be applicable in infants who are otherwise well nourished.[68]

5. Cancer

The beneficial effects of yogurt consumption on reduced cancer incidence is not established. The article published by van't Veer and colleagues[69] is often quoted as proof of a link between yogurt consumption and a low incidence of breast cancer. Based on a daily food consumption questionnaire of newly diagnosed breast cancer patients or a group of healthy women (control), they concluded that fermented milk products (yogurt, buttermilk, Gouda cheese) may have a protective effect against breast cancer. Two more recent studies came to different conclusions about the effect of yogurt (fermented milk products) on colorectal cancer.[70,71]

The work of Goldin and Gorbach[42] and Goldin and colleagues[72] supports the idea that the *Lb. acidophilus* found in yogurt may impact on marker enzymes related to cancer. They found two to sixfold reductions in the activities of β-glucuronidase, nitroreductase, and azoreductase enzyme activities after 4-week supplementation with 10^9 to 10^{10} viable *Lb. acidophilus*. These bacterial enzymes produce proximal carcinogens from procarcinogens in the lower intestine. This work together with positive *in vitro* and experimental animal results suggest that the bacteria in yogurt and other probiotic bacteria deserve further study.[73–76]

Other work on the cancer-fighting properties of yogurt has centered on the isolation and identification of bioactive peptides. Caseins found in milk are themselves antimutagenic; totally hydrolyzed caseins are not. A wide variety of peptides of various lengths are formed from milk during fermentation, or in the stomach during the digestion of yogurt.[77–79] The peptides vary in length, physiological activity, and ability to be absorbed into the bloodstream from the GI tract. These relatively short peptides have a wide variety of activities *in vitro* and *in vivo,* including antimutagenic and antitumor properties that may explain beneficial effects of yogurt toward cancer. In addition, milk peptides have been shown to have opioid, antihypertensive, immunomodulating, antibacterial, antiaggregating, and antithrombotic effects.

6. A Vehicle for Other Nutrients

The popularity of yogurt has prompted several studies that have investigated the feasibility of using yogurt as a vehicle for other important nutrients that normally would not be of importance in yogurt. Fernandez-Gartcia and colleagues[80] have recently shown that oat fiber can be added to plain yogurt while still maintaining commercially acceptable sensory qualities.

In spite of the potential problems of off-flavor and encouragement of growth of contaminating bacteria, Hekmat and McMahon[81] were able to produce a yogurt with up to 40 mg/kg of added iron that was acceptable particularly to untrained consumers.

B. Kefir

Kefir is a fermented milk drink that has its origins in the Caucacus Mountains of the former U.S.S.R. Traditionaly it was made from goat's or cow's milk that was stored in skin bags. Over time a mass of bacteria/yeast/protein/carbohydrate precipitated out from the drink that was used to innoculte new milk. This mass is called kefir grains, and it is the grains that give kefir its texture, taste, and possible health benefits. Kefir has a long oral tradition for its health-promotion properties in Eastern Europe and has only recently been produced in North America on a commercial scale.[82] A product of the fermentation process is CO_2 gas, which continues to be produced after packaging and results in a thick drink with a "sparkling" mouth-feel when consumed. This has prompted some to refer to kefir as the "champagne of dairy products."

Unlike yogurt, which requires only two well-defined bacteria for production, the microbiology of kefir is much more complex and has been shown to vary from country to country, thus making a comparison of its properties difficult (Table 25.7). Changes that occur during fermentation promote the growth of certain microorganisms, while reducing the growth of others. Therefore, the composition and properties of the grains may not be totally applicable to the

TABLE 25.7
Microorganisms Reported to be Found in Kefir and Kefir Grains[83,90–92]

***Lactobacillus* Bacteria**	***Lactococcus* Bacteria**
Lb. acidolphilus	*L. lactis* subsp. *lactis*
Lb. johnsonii	*L. lactis* subsp. *cremoris*
Lb. kefirgranum	*L. lactis* subsp. *lactis biovar diacetylactis*
Lb. helveticus	
Lb. delbrueckii subsp. *bulgaricus*	
Lb. kefiranofaciens	**Leuconostoc Bacteria**
Lb. casei	*Ln. lactis*
Lb. zeae	*Ln. mesentroides* subsp. *mesentroides*
Lb. rhamnosus	*Ln. mesentroides* subsp. *cremoris*
Lb. plantarum	*Ln. mesentroides* subsp. *dextranicum*
Lb. brevis	
Lb. buchneri	**Other Bacteria**
Lb. fermentum	*Streptococcus thermophilus*
Lb. kefir	
Lb. parakefir	

Yeasts

Saccharomyces Yeast	***Kluyveromyces Yeasts***
S. cerevisiae	*K. marxianus* var. *marxianus*
S. unisporus	*K. marxianus* var. *lactis*

Other Yeasts
Candida kefyr
Torulaspora delbrueckii
Geotrichum candidum Link

The microflora of kefir drink is not merely a dilution of the microflora of the kefir grains, because during the production process conditions may favor the growth of certain bacteria and discourage the growth of others.

1. Fabrication

Three different types of kefir drink are produced — traditional kefir, Russian-type kefir, and industrial kefir — depending on whether the grains or a mother culture from the grains is used to innoculate pasteurized milk.[82,83] No studies have been published that compare the properties (health benefits or otherwise) of kefir produced by different processes. Incubation is carried out at 20 to 22°C for 18 to 20 h until a specific pH is reached, and then packaged, or a maturation period can be introduced.[83] The grains themselves have a strong yeasty taste, and, therefore, using a mother culture or sieving out the grains may be ways of making a product closer to buttermilk in taste.[84,85] A double-fermentation process was proposed by Marshall and Cole[86] as a way of making the taste of traditional kefir more acceptable to the consumer.[87] Today, commercial kefir is only produced from cow's milk, but other milks can be fermented with kefir grains.[88] In terms of volume, kefir is the dairy product most consumed in Russia.

Studies of the microflora of kefir grains led to the formation of less complex starter cultures that could replace kefir grains. A method using as few as four bacteria and one yeast type has been reported as being used to produce "kefir."[84] However, apart from very gross characteristics, there

is little reason to believe that the two beverages (produced from traditional grains or starter cultures) are the same.[89]

2. Health Benefits

The (Western) scientific literature to support the beneficial health claims for kefir is not extensive and few human feeding trials using kefir have been carried out. The Russian literature lists peptic ulcers, biliary tract diseases, chronic enteritis, bronchitis, and pneumonia as all being treated with kefir.[82] Kefir is included as a regular part of hospital diets, is a recommended food for nursing mothers, and is often used as a first weaning food for babies in Russia. Both kefir grains and the drink itself have been shown to have antitumoral, antibacterial, and antifungal properties that may explain the diverse list of diseases and infections it is used to treat.[93] In the tests carried out by Cevikbas and colleagues[93] the kefir grains were more effective than the drink. Osada and co-workers[94] reported the isolation of a sphingomyelin species isolated from kefir grain which they showed enhanced interfereon-β production in a human cancer cell line that had been challenged with a chemical inducer. They concluded that this sphingomyelin could be important in viral diseases.

Several Japanese publications in experimental animals have indicated anticancer properties of kefir for a variety of cancers.[95–100] These studies have used kefir grains or kefir grain polysaccharide both to prevent the onset of cancer, if given before a cancer challenge, or to slow the growth and spread of cancer, if given after the cancer challenge. However, to date such experiments have not been carried out in humans.

Vujicic and colleagues[101] incubated milk samples with kefir grains from six different sources and showed that, after 24 h, between 22 and 63% of the cholesterol originally in the milk was assimilated; they concluded that the grains possessed a cholesterol-degrading enzyme system. In a recent study in which hypercholesterolemic men were given 500 ml of kefir daily for 1 month, no changes (compared with values obtained after one month of milk feeding) were measured in serum cholesterol levels or cholesterol metabolism, in spite of the fact that a reference organism (*Lb. brevis*) could be found in fecal samples and changes in fecal volatile fatty acids were found.[11,27,102]

V. FERMENTED VEGETABLES AND OTHER FOODS

Fermented vegetables are known throughout the world. Fermentation is often viewed as an effective method to preserve vegetables, without regard to any health benefits. Reddy and colleagues.[103] list 23 legume-based fermented foods, the majority of which are soybean products. Only natto, a popular fermented soybean product from Japan, is mentioned as encouraging the growth of *Bifidobacterium* in animals.[104] Most reports of fermented vegetables have centered on the organism(s) used to produce the fermentation and on any increases in the protein, amino acid, and vitamin concentrations of the final product, not any possible probiotic effects.[105–107]

Various other foods have been tested as possible vectors to carry probiotic bacteria. The verification of the efficacy of these products has not been the focus to date, but, rather, investigation of whether the bacteria will survive in the food matrix and during processing and storage. Cheddar cheese containing *Enterococcus faecium,*[108] *B. longum* in frozen yogurt,[109] and *B. longum, B. infantis,* and *B. brevi,*[30] *L. acidophilus,* and *B. bifidum*[107] in ice cream are examples.

Lee and Salminen[5] predicted that probiotic infant formulae, baby food, fermented fruit juices, fermented soy products, cereal-based products, as well as disease-specific products, are products of the future.

VI. THE FUTURE FOR PROBIOTICS AND PREBIOTICS

The market for probiotic and prebiotic products will continue to grow as our knowledge of the intestinal microflora and its role in the maintenance of health and disease resistance advances. Food

manufacturers will have to be able to produce products that maintain viable bacteria up until the time of consumption and in many cases will also have to provide encapsulation or other protective mechanisms for the live microorganisms in their products to be able to deliver bacteria to the correct site of action in the GI tract.

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26 Lecithin and Choline: New Roles for Old Nutrients

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I. INTRODUCTION

Once topics of "health food lore," lecithin (phosphatidylcholine) and its main component, choline, are important nutrients found in a wide variety of foods and available as supplements. A quaternary amine, choline has long been recognized as an essential nutrient for a number of animal species, and more recent evidence suggests it is essential for humans as well. The Food and Nutrition Board, which has established and revised the Recommended Dietary Allowances (RDAs) for nutrients since 1941, established the first Dietary Reference Intakes for choline in 1998.[1]

Beyond its function as an essential nutrient, some research suggests choline as well as lecithin may play roles in other health and disease states, including cardiovascular disease, reproduction and development, memory, and physical performance. Underlying these potential benefits are the functions of lecithin and choline in methyl group metabolism, cholesterol transport, acetylcholine synthesis, and cell signaling.

II. HISTORICAL PERSPECTIVE

Choline was first recognized as present in mammalian tissues by Strecker[2] in 1862. Its importance as an essential nutrient was first realized in studies on insulin in the 1930s.[3,4] Depancreatized dogs maintained on insulin therapy developed fatty livers and died. Administration of raw pancreas

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prevented fatty liver due to its choline content in the form of lecithin. As a result, choline and other substances that prevented fatty liver were designated "lipotropic."

Research over the years established choline as an essential nutrient in many animal species, including primates. In addition to fatty liver, choline deficiency has been shown to cause abnormal kidney function, infertility, growth impairment, bone abnormalities, decreased hematopoiesis, and hypertension.[5]

Health food advocates have espoused the benefits of lecithin for decades. In the 1970s some suggested, often with little evidence, it was beneficial for conditions ranging from heart disease to baldness.[6] Articles in health food publications in the 1980s discussed lecithin as a treatment for memory loss, neurological disorders, heart disease, and gall bladder disease.[7] Although early research on lecithin suggested some such benefits, little was known about its actual role in nutrition and health. As a result of research over the last 10 to 15 years in molecular and cell biology, neuroscience, and nutrition, much has been learned about lecithin and choline and their roles in health and disease.[8–11]

III. DIETARY SOURCES AND INTAKE

In foods, choline occurs mostly in the form of lecithin (phosphatidylcholine) but may also be in the free form or as a component of other phospholipids such as sphingomyelin (Table 26.1). Chemically, choline is hydroxyethyl trimethyl ammonium hydroxide (Figure 26.1). Because it is a zwitterion (having both a positive and negative charge), choline supplements are in the form of various salts such as choline chloride, choline bitartrate, and choline dihydrogen citrate.

Lecithin contains about 13% choline by weight (Figure 26.1). It is a member of the phospholipid family and, as such, is similar in structure to triglycerides except the fatty acid esterified in the sn-3 position of glycerol is replaced with choline linked by a phosphate ester bond. Lecithin is also a zwitterion, having a positive charge on the N atom of choline and a negative charge on an O atom of the phosphate group. The polar choline portion and nonpolar fatty acid portion of lecithin make it a very effective emulsifying agent and useful in various drug-delivery systems.[12]

"Commercial lecithin," used in nutritional supplements, is actually a mixture of phosphatidylcholine and other phospholipids extracted from soybeans. Commercial lecithin comprises about 3% of soybean oil which, in turn, is about 18% of the weight of soybeans. Granular commercial lecithin contains about 23% phosphatidylcholine; typical lecithin capsules provide about 15%. One tablespoon of lecithin granules provides about 1725 mg of phosphatidylcholine and 250 mg of choline, a little less than the content in an egg. One capsule provides about 180 mg of phosphatidylcholine and 24 mg of choline (see Table 26.1).

Although lecithin and choline can be found in a wide variety of foods, many of the richest sources are foods also high in cholesterol and fat. For example, as shown in Table 26.1, egg yolks and organ and other meats are good sources of lecithin and choline; grains, fruits, and vegetables are much poorer sources.

Because many Americans have decreased consumption of fatty foods, their intake of lecithin and choline may be less than optimal.[13] It has been estimated that healthy adults in the United States consume about 6 g/day of lecithin and 0.6 to 1.0 g/day of choline.[5] These estimates, however, were based on food consumption data in the 1970s. Since then, Americans cut their total fat intake by about 15%, largely by decreasing their consumption of foods such as eggs by about 20%, organ meats by about 50%, and red meat by about 48% — all major sources of choline and choline phospholipids.[14,15]

Although both lecithin and choline are available as supplements, lecithin appears to provide a more bioavailable source of choline than do choline salts. This has been shown in studies in which plasma levels of choline were measured after individuals consumed equal amounts of choline in the form of lecithin or choline salts.[16–18] For example, lecithin sustained plasma choline at a higher level for a longer period of time compared with choline chloride, as shown in Figure 26.2.

TABLE 26.1
Choline and Choline Phospholipid Content of Some Common Foods[49,134,135]

Food (serving size)	Free Choline mg/serving	Lecithin (phosphatidylcholine) mg/serving	Sphingomyelin mg/serving	Total Choline[a] mg/serving
Apple (1 medium)	0.39	29.87	1.51	4.62
Banana (1 medium)	2.85	3.26	1.66	3.52
Beef liver (3.5 oz)	60.64	3,362.55	134.68	532.28
Beef steak (3.5 oz)	0.78	466.12	36.84	68.75
Butter (1 pat)	0.02	6.80	1.67	1.18
Cauliflower (1/2 cup)	6.79	107.06	6.66	22.15
Corn oil (1 T)	0.00	0.13	0.05	0.03
Coffee (1 cup)	18.59	2.05	2.96	19.29
Cucumber (1/2 cup)	1.18	3.06	1.02	1.74
Egg (1 large)	0.22	2,009.80	81.90	282.32
Ginger ale (12 oz)	0.08	1.11	0.78	0.34
Grape juice (6 oz)	8.99	2.11	0.66	9.37
Human milk (1 cup)	2.10	27.08	31.83	10.29
Iceberg lettuce (1 oz)	8.53	2.86	1.02	9.06
Infant formula (1 cup)	6.55	23.77	8.76	11.00
Lecithin supplement (commercial, powdered; 1 T, 7.5 g)	0.00	1725.00	0.00	250.00
Lecithin supplement (1 soft gel capsule)	0.00	180.00	0.00	24.22
Margarine (1 pat)	0.02	1.74	0.05	0.26
Milk (whole, 1 cup)	3.81	27.91	14.57	9.64
Orange (1 medium)	2.91	53.03	2.45	10.40
Peanut butter (2 T)	12.96	97.39	0.21	26.09
Peanuts (1 oz)	13.24	107.35	1.59	27.91
Potato (1 medium)	5.95	25.97	2.12	9.75
Tomato (1 medium)	5.50	4.94	2.87	6.58
Wholewheat bread (1 slice)	2.52	6.57	0.20	3.43

[a] Total choline in each food is the sum of free choline and choline from choline-containing phospholipids.

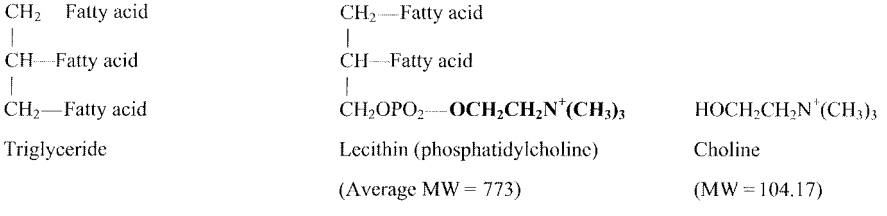

FIGURE 26.1 Molecular structures for a triglyceride, lecithin, and choline.

Lacithin is probably more bioavailable than choline salts because less of the choline in lecithin is converted to di- and trimethylamine by intestinal bacteria. About 60% of orally ingested choline salts is lost this way.[19] However, ingesting an equal amount of choline in the form of lecithin produced only about a third as much trimethylamine.[20] High amounts of trimethylamine in the gastrointestinal tract can produce an offensive, fishy body odor.

Choline and lecithin are generally safe and nontoxic. In a report to the Food and Drug Administration (FDA), an expert panel suggested a maximum tolerated dose of 16 to 20 g/day (about 30 times dietary intake levels) for choline, noting adverse effects of nausea, sweating,

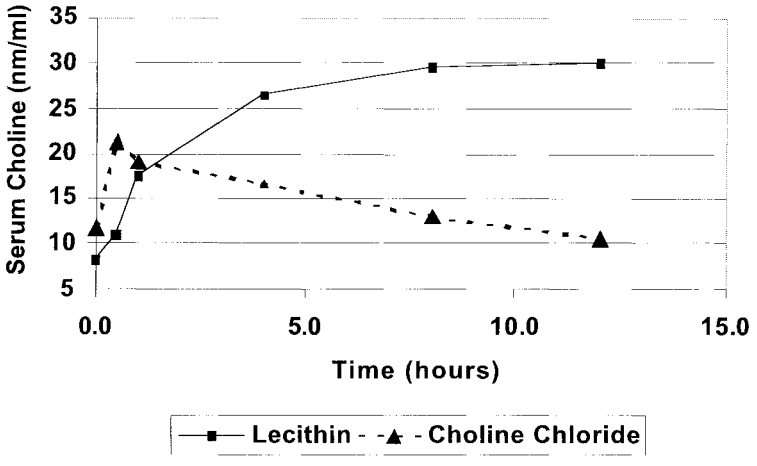

FIGURE 26.2 The effects of lecithin and choline chloride on serum choline levels in normal subjects. (Adapted from Hirsch, M.J. et al., *Metabolism*, 27: 953–960, 1978.)

anorexia, fishy body odor, and cardiac arrhythmias at higher levels. No maximum tolerated dose for lecithin was specified, although the panel noted that most individuals appear to tolerate 40 g/day of phosphatidylcholine (85%) without adverse effects. Abdominal bloating, diarrhea, and vomiting have been associated with higher doses.[21] Commercial lecithin, phosphatidylcholine, choline bitartrate, and choline chloride are all generally recognized as safe (GRAS) (21 CFR §182,184).

IV. METABOLIC ROLES

Choline, a component of a number of important metabolites (Table 26.2), functions in five major metabolic pathways (Figure 26.3):

- Acetylation to the neurotransmitter acetylcholine;
- Irreversible oxidation to betaine, a methyl group donor, which participates in homocysteine metabolism;
- Phosphorylation to phosphocholine, the first step in the cytidine diphosphate pathway for generating lecithin;
- Substitution through base exchange for serine, inositol, ethanolamine, or other head groups on phospholipids to generate lecithin (not shown); and
- Generation of sphingomyelin as a result of the transfer of phosphocholine from lecithin to ceramide (not shown).

Some of these metabolites and pathways will be discussed later as they relate to various health and disease states.

Additionally, lecithin is synthesized by the sequential methylation of phosphatidylethanolamine by phosphatidylethanolamine-*N*-methyltransferase, using *S*-adenosylmethionine as the methyl donor (see Figure 26.3). This pathway is important in the brain and liver because it contributes choline for acetylcholine synthesis and is the only way to generate new choline molecules.[8] Both the cytidine diphosphate and base exchange pathways simply redistribute existing choline molecules.

Choline, folate, vitamin B_{12}, and methionine all participate in methyl group and homocysteine metabolism. As the synthetic precursor of lecithin, phosphatidylethanlolamine is a methyl acceptor from *S*-adenosylmethionine. The oxidized intermediate of choline, betaine, can donate a methyl

TABLE 26.2
Structures and Functions of Major Choline Metabolites

Name	Structure	Function
Choline	$(CH_3)_3N^+CH_2CH_2O^-$	Methyl group metabolism; constituent of various compounds (see below)
Lecithin (phosphatidylcholine)	CH_2--$OCOR_1$ | CH--$OCOR_2$ | CH_2OPO_2--$\mathbf{OCH_2CH_2N^+(CH_3)_3}$	Membrane phospholipid, choline reserve; provides diacyglycerol, fatty acids, and phosphoryl-choline for cell signaling
Acetylcholine	$\mathbf{(CH_3)_3N^+CH_2CH_2}$--$COOCH_3$	Neurotransmitter used by cholinergic nerves
Sphingomyelin	$HOCHC=CH(CH_2)_{12}CH_3$ | CH--$NHCOR_1$ | CH_2OPO_2-$\mathbf{OCH_2CH_2N^+(CH_3)_3}$	Neural membrane phospholipid; participates in cell signaling
Platelet-activating factor	$CH_2OCO(CH_2)_{15}CH_3$ | CH $OCOCH_3$ | CH_2OPO_2--$\mathbf{OCH_2CH_2N^+(CH_3)_3}$	Activation of platelets, inflammatory and immune responses, egg implantation and induction of labor
Plasmalogen	$CH_2OCH=CHR_1$ | CH $OCOR_2$ | CH_2OPO_2--X	Found in sarcolemma, the membrane of cardiac muscle; breakdown may contribute to sequelae of myocardial infarction

R = alkyl chain of a fatty acid; X = choline or ethanolamine.

group to homocysteine, forming methionine. Methionine can be converted back to homocysteine as well as contribute to choline synthesis. Since high levels of homocysteine increase the risk for cardiovascular disease,[22] an adequate intake of choline may be important in reducing risk, as discussed later. Thus, methyl group and homocysteine metabolism are complex and the requirement for participating nutrients are interdependent. This is one reason establishing a precise human requirement for choline has been difficult.

Methotrexate, a drug used in treating cancer, psoriasis, and rheumatoid arthritis, limits the availability of methyl groups by inhibiting the enzyme dihydrofolate reductase, which is involved in folate metabolism. Under these circumstances, choline becomes the only other source of methyl groups. Hepatic levels of choline metabolites decrease after methotrexate treatment, probably reflecting their increased use as methyl donor sources, and can lead to fatty liver. Supplemental choline reverses fatty liver caused by methotrexate therapy.[8]

V. CHOLINE PHOSPHOLIPIDS IN CELL SIGNALING

Residing in cell membranes, lecithin and other phospholipids also serve as sources of second messengers in cell signaling, the process by which hormones and other substances transmit signals from the outside of a cell to the interior. Without this essential process cells could not properly grow, replicate, or absorb and use nutrients for energy.[10,11]

Cell signaling is the focus of a great deal of current research. In 1994, Drs. Alfred Gilman and Martin Rodbell from the United States were awarded the Nobel prize in Physiology or Medicine for their research on cell signaling. They focused on the first step in this process,

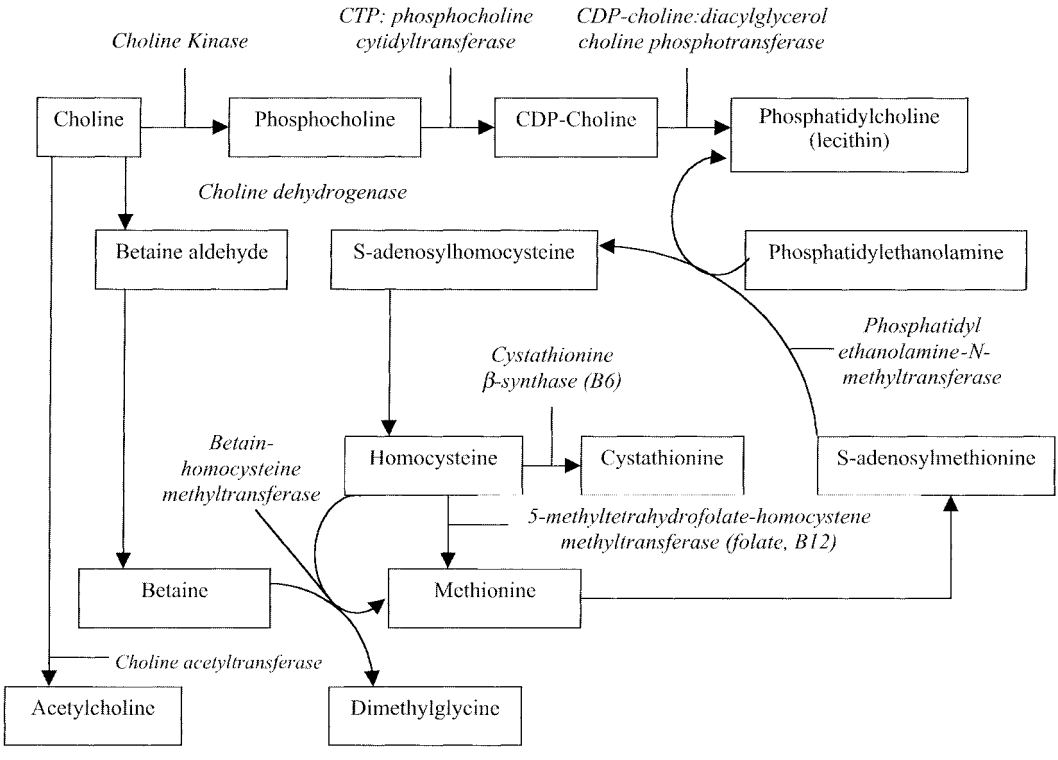

FIGURE 26.3 Choline metabolism.

which involves activation of enzymes known as G proteins in cell membranes by some external stimulus (for example, a hormone). The activated G protein stimulates one of a number of phospholipases that break down lecithin or another target phospholipid in the membrane. The breakdown products in turn activate a key enzyme, protein kinase C (PKC). This central, regulatory enzyme triggers a cascade of subsequent reactions that can affect a myriad of processes including cell growth, metabolism, nutrient uptake, ion transport, muscle contraction, and even programmed cell death (apoptosis).

A key question driving much research is how the plethora of possible cell signals — such as different hormones, ions, and nutrients — translate into different, specific messages inside the cells. Part of the answer lies in the diversity of the signaling process. Different signals stimulate different G proteins, which activate different phospholipases and produce different phospholipid breakdown products. For example, lecithin is broken down to a free fatty acid and lysophosphatidylcholine by phospholipase A2, to 1,2 diacylglycerol and phosphocholine by phospholipase C, and to phosphatidic acid and choline by phospholipase D. Each of these breakdown products (except probably choline) has different effects on PKC. Other phospholipids, such as sphingomyelin, phosphatidylinositol, and phosphatidylethanolamine, produce still other second messengers. Finally, at least ten different forms of PKC exist, each of which may serve different functions. These many possibilities probably account for at least some of the broad range of functions that lecithin, other phospholipids, and PKC mediate.[10,11]

Much remains to be learned about the precise functions of the different signaling pathways and how they are controlled. However, it is becoming clear that disruptions in the signaling process can lead to abnormal states such as cancer and Alzheimer's disease, as discussed later. Nevertheless, given the multiple functions of choline and lecithin in methyl group metabolism and cell signaling, it is not surprising these nutrients are involved in many areas of health and disease prevention (Table 26.3).

TABLE 26.3
Roles of Choline and Lecithin in Health and Disease States

Health or Disease Area	Evidence for Roles of Lecithin and/or Choline
Liver function	Choline is required for normal liver function. Choline deficiency causes: • Fatty liver and liver cancer in animals • Fatty liver in patients given total parenteral nutrition • Abnormal liver function in healthy adult men within a few weeks Lecithin protects against alcohol cirrhosis (shown in primate studies; clinical trials currently in progress)
Cardiovascular disease	• Choline facilitates the metabolism of homocysteine, which increases cardiovascular disease risk • Lecithin lowers serum cholesterol levels • Lecithin is an obligatory component of VLDL and HDL
Reproduction and development	Choline: • Plays an important role in brain development and learning (required in FDA-approved infant formulas) • Can affect the status of folate, which is recommended for women of child-bearing age to prevent neural tube defects • Is a component of platelet activating factor, which plays a role in egg implantation and the induction of labor • Enhances sperm motility
Physical performance	Choline supplementation improves performance in various endurance sports
Memory	Lecithin and choline improve memory and learning in animals and short-term memory in humans

VI. CHOLINE, LECITHIN, AND LIVER FUNCTION

As mentioned above, the requirement of choline for normal liver function has been known for more than 60 years and is the most recognized role of choline in health and disease. Numerous animal studies have shown a choline-deficient diet promotes liver carcinogenesis.[23–28] The disease begins with early signs of lipid accumulation in the liver because lecithin is required to synthesize very low density lipoproteins (VLDLs), the liver's major export route for triglycerides. Indeed, fatty liver is one of the classic signs of choline deficiency in animals and is observed in humans fed by total parenteral nutrition (TPN).[5]

When choline deficiency has been prolonged in animal studies, fatty liver is followed by cell necrosis and tissue fibrosis and progresses to cirrhosis and carcinoma.[29] Choline deficiency has been shown to result in liver cancer in the absence of exposure to carcinogens[28,30] and to promote cancer in the presence of various carcinogens.[29,31] Further, an excess of choline and/or methionine protects against hepatocarcinogenesis in mice exposed to a carcinogen (aflatoxin B1).[32]

A number of potential mechanisms have been proposed for the carcinogenic effect of choline deficiency, including increased cell death followed by increased cell proliferation and regeneration; decreased DNA methylation and repair; increased lipid peroxidation and free radical damage; decreased methylation and detoxification of carcinogens; and formation of excessive amounts of diacylglycerol (DAG) from lecithin breakdown (to supply the missing choline), which overstimulates PKC.[33] Various lines of evidence support each of these mechanisms and some or all may be at work.[34] The latter proposal is of particular interest in light of the recently recognized role of choline phospholipids in regulating PKC, and is supported by recent research.

In one study,[33] rats fed a choline-deficient diet had increased levels of DAG in liver plasma membranes at 1 and 6 weeks and decreased levels at 27 and 52 weeks. Plasma membrane PKC activity was increased at all four time points. After 1 year, 69% of animals exhibited atypical hepatic

foci and 23% had hepatocarcinomas. None of the controls had atypical foci or cancer. These results suggest choline deficiency disturbed the PKC-mediated transmembrane signaling in the liver and contributed to development of hepatic cancer.

Disruptions in choline phospholipid PKC signaling mechanism may also be involved in other types of cancer, such as colon cancer.[35] A number of lecithin analogues have been used in clinical trials as cancer therapeutics.[36,37]

Lecithin or choline is essential to liver function in humans as well. Healthy adult males fed a semisynthetic diet devoid of choline for a few weeks developed markedly diminished blood levels of choline and elevated plasma levels of alanine aminotransferase (ALT, also known as glutamate pyruvate transaminase, or GPT), an early sign of liver dysfunction.[38] Individuals undergoing long-term TPN therapy develop fatty infiltration of the liver and hepatocellular damage.[39] Supplemental choline (in the form of lecithin) has reversed fatty liver in these patients.[40]

Research by Lieber and colleagues suggests lecithin may also protect the liver independently of providing choline. In two studies, baboons were fed diets high in alcohol with or without added lecithin for up to 8 years. About 80% of those not provided supplemental lecithin developed liver fibrosis or cirrhosis, characterized by collagen accumulation, fibrosis, and scarring. However, no animals fed lecithin developed fibrosis or cirrhosis.[41,42] An earlier study showed choline did not provide this protective effect.[43] *In vitro* studies showed lecithin increased the activity of hepatic collagenase, suggesting lecithin helps prevent alcohol-induced fibrosis and cirrhosis partly by promoting collagen breakdown.[42] Clinical trials of lecithin supplementation in alcoholic adults are under way.

VII. CHOLINE AND LECITHIN IN REPRODUCTION AND DEVELOPMENT

Lecithin and choline play a number of roles in reproduction and development. Choline is widely recognized as important in brain and mental development of both the fetus and infant.[9,44] After choline is fed to pregnant rats, their offspring show significantly better memory in maze tests than rats whose mothers were not fed choline.[45] The improved memory is maintained even after the rats grow old and at a level comparable to that of much younger rats. Other rat studies have shown choline-induced memory improvement is associated with short- and long-term changes in certain brain nerve cells and substances.[46]

Because choline is so important in development, it is favorably transported across the placenta from the mother's bloodstream to the fetus by a 1:14 ratio.[47,48] Similarly, the choline concentration in mother's milk is over 100 times the level in the maternal bloodstream.[49] Thus, the requirement for choline appears to be especially high in women during pregnancy and lactation. Platelet-activating factor, another choline phospholipid (see Table 26.2), is involved in implanting the egg in the uterine wall,[50] fetal maturation, and inducing labor.[51]

In the United States, infant formulas approved by the FDA are required to contain at least 7 mg of choline per 100 kcal (16.7 μg/kJ), based on the choline content in human milk.[52,53] Colostrum and transitional milk contain approximately 1400 μmol/l (169 mg/l) choline moiety (about 100 μmol/l free choline, 300 μmol/l choline in phosphatidylcholine and sphingomyelin,[49] and 1000 μmol/l choline as glycerophosphocholine and phosphocholine[54]). Based on an energy requirement of 418 kJ/kg body weight (BW)/day (100 kcal/kg/day) for an infant and a caloric density of 2.76 kJ/ml (0.66 kcal/ml) for human milk, a breast-fed infant drinking about 150 ml milk/kg BW/day would ingest approximately 210 μmol (22 mg) of choline/kg BW/day. Infant formulas provide from 700 to 1800 μmol/l (10.9 to 28.1 mg/150 ml) total choline.[54]

As described above, choline and folic acid both participate in methyl group metabolism, which is necessary for normal DNA methylation and homocysteine metabolism. These functions may relate to the relationship between inadequate folic acid intakes and neural tube defects (NTDs), devastating abnormalities affecting 2500 to 3000 U.S. births each year.[55] Indeed, mothers with NTD neonates had higher circulating homocysteine levels than normal mothers.[56,57] In 1992, the Centers

for Disease Control recommended all women of child-bearing age take 400 μg/day of folic acid to help prevent these defects.[58] As an additional preventive measure, the FDA now requires folic acid supplementation of a broad variety of grain foods.[59]

NTDs are complex disorders, probably involving other nutrients as well. In particular, since folic acid and choline work together in methyl group metabolism, an inadequate choline intake might raise homocysteine levels, as described earlier. Inadequate choline could also lower folic acid levels in women, endangering a current or future pregnancy. This is supported by animal studies showing choline deficiency caused below-normal liver folic acid levels.[60] Thus, like folic acid, choline may play a role in preventing NTDs.

Lecithin may also play a role in male fertility. *In vitro* studies showed lecithin restored normal structure and movement to abnormal sperm cells[61] and nearly doubled acrosomal response, an indicator of the ability of sperm to enter and fertilize an egg.[62] The researchers noted lecithin might be useful in enhancing fertility of men with decreased acrosomal responsiveness.

VIII. CHOLINE AND LECITHIN IN CARDIOVASCULAR HEALTH AND DISEASE

Choline and lecithin also play a number of roles in cardiovascular function and may lower the risk of cardiovascular disease (CVD). As noted above, choline participates in the metabolism of homocysteine, a sulfur-containing amino acid that increases CVD risk. Lecithin can lower plasma cholesterol levels and is a key component of various circulating lipoproteins that transport fat and cholesterol. Choline is also the key component of plasmalogen, an important phospholipid in the cardiac muscle cell membrane.

The link between homocysteine and CVD was first described by Dr. Kilmer McCully over 30 years ago.[63] He studied individuals with a rare, inherited disorder in which an enzyme that metabolizes homocysteine, cystathionine β-synthase, is deficient or defective. These individuals have very high blood levels of homocysteine and usually die from CVD before adulthood. McCully proposed that even moderately elevated levels of homocysteine in normal individuals might increase CVD risk. At the time, however, most scientists focused on the effects of fat and cholesterol.

More recent evidence supports McCully's theory. Studies in large population groups have shown high homocysteine levels increase the risk of heart attack,[64] clogged carotid arteries (a risk for strokes),[65] and myocardial infarction.[66] A review of 27 studies in this area strongly linked high homocysteine levels with increased CVD risk.[22] The mechanisms of the atherogenic effects of homocysteine are not known but may result from its promotion, in blood vessels, of low-density lipoprotein (LDL) aggregates, smooth muscle cell hyperplasia and fibrosis, and deposition of calcium and sulfated materials.[67]

Homocysteine is made in the body from the essential amino acid methionine (see Figure 26.3). Researchers often determine abnormal homocysteine metabolism in patients by determining their plasma homocysteine levels while fasting or after a standard methionine load test or by measuring urinary homocysteine levels.

Homocysteine is metabolized by three possible pathways (see Figure 26.3). In the B_6 pathway, homocysteine is metabolized by the vitamin B_6-dependent enzyme cystathionine β-synthase. It can also be converted back to methionine in the folic acid pathway by the enzyme methylfolate-homocysteine methyltransferase, which also requires vitamin B_{12}. Finally, in the choline pathway (active only in the liver in humans), homocysteine is converted to methionine by betaine-homocysteine methyltransferase, which uses betaine, an oxidized form of choline readily made in the body.[68] Defects in enzymes involved in any of these pathways, deficiencies of the corresponding vitamins, as well as various disease states and drug therapies can elevate plasma homocysteine levels.[69]

Regardless of the actual defect, therapy with vitamin B_6, choline (or betaine), and/or folic acid can reduce homocysteine levels, though not always. Indeed, the circumstances under which one therapy will or will not work are not always clear. For example, large doses of vitamin B_6 reduce fasting homocysteine levels in about 50% of patients with cystathionine β-synthase deficiency.[69]

However, some studies show this therapy usually only slightly reduces homocysteine levels after a methionine load test.[70] Still others report vitamin B_6 therapy has minimal effect on fasting levels of homocysteine but a more dramatic effect after methionine loading.[71]

In any case, betaine and folic acid have also successfully lowered homocysteine levels during fasting and after methionine loading.[72-75] For example, 6 g/day of betaine significantly reduced homocysteine levels both in patients nonresponsive[76] and responsive[77] to vitamin B_6 therapy.

Dudman and colleagues[78] treated CVD patients with hyperhomocysteinemia with either choline (2 g/day for 1 week), betaine (1.7 g/day for 1 week), vitamin B_6 (100 mg/day [5000% U.S. RDA] for 2 weeks), or folic acid (5 mg/day [1250% U.S. RDA] for 2 weeks). Homocysteine levels following a methionine load test were successfully reduced by 45 to 60% in 16 of 19 patients. All four supplements worked about equally well. This group also published earlier reports that betaine lowered plasma homocysteine levels.[79] Other studies have found choline or betaine effectively lowered homocysteine levels when given in combination with therapeutic doses of vitamin B_6 and/or folic acid.[71,80,81] However, betaine did not lower homocysteine levels in patients with chronic renal failure maintained on folate supplementation.[82]

It should be noted that since the above intervention studies were in CVD patients with hyperhomocysteinemia, they employed high therapeutic doses of vitamins. However, as reviewed by Boushey and colleagues,[22] other studies have shown strong, inverse correlations between plasma levels of homocysteine and vitamins B_6 and folic acid, which reflects dietary intake of the vitamins. Dietary choline deficiency causes arteriosclerosis in rats,[83] apparently due to hyperhomocysteinemia.[63] This suggests adequate dietary intakes (not necessarily high, therapeutic doses) of lecithin or choline may also provide important protection against high homocysteine levels and, therefore, lower CVD risk.[84] This is an area that deserves further study.[85]

Other evidence suggests lecithin may also reduce CVD risk by contributing cholesterol-lowering polyunsaturated fats, inhibiting intestinal absorption of cholesterol, increasing the excretion of cholesterol and/or bile acids, and favorably affecting lipoprotein profiles.[86] Supplemental lecithin has lowered levels of serum total- and low-density cholesterol (LDLs), and increase levels of high-density cholesterol (HDLs) in adults with hyperlipidemia[87] and type II diabetes.[88] It has even shown these effects when applied topically to the skin in humans[89] and in hypercholesterolemic animals.[90]

Lecithin may also act favorably on serum HDLs, which decrease CVD risk. Lecithin is a required substrate for lecithin-cholesterol acyltransferase, an enzyme that esterifies cholesterol, sequestering it into the lipophilic interior of the HDL, enabling it to pick up additional unesterified cholesterol on its more polar outer surface. O'Brien and Andrews[86] found that soy lecithin increased HDL levels more than did egg lecithin or an equivalent amount of triglyceride containing comparable fatty acids. Further research is needed to determine the precise mechanism for these beneficial effects of lecithin on lipoproteins.

Finally, choline is a key component of plasmalogen, a phospholipid found at high levels in the sarcolemma, the cell membrane of heart muscle. The adverse sequelae of acute myocardial ischemia may be a result of plasmalogen breakdown during an ischemic attack. Support for this comes from studies demonstrating a relatively new phospholipase A2 (PLA2) enzyme in the sarcolemma. PLA2 hydrolyzes plasmalogen to produce a free fatty acid (usually arachidonate). Although most PLA2 enzymes are believed to be calcium dependent, the sarcolemma PLA2 is calcium independent and activated by adenosine triphosphate (ATP). It has been suggested that, during an ischemic attack, ATP levels drop and then increase, activating this specific PLA2 and increasing arachidonate levels, which may alter membrane permeability and ion transport, thus precipitating abnormal heart contractions.[91]

IX. CHOLINE AND LECITHIN IN MEMORY FUNCTION

A good deal of evidence from animal studies shows lecithin and choline improve memory and learning.[92,93] As noted above, rats born to mothers supplemented with lecithin demonstrate improved

learning.[44,45] These benefits are probably due to the conversion of lecithin and choline to the neurotransmitter acetylcholine.

Studies in humans have also suggested these nutrients can improve learning and memory,[94–96] although not all have shown benefits from lecithin therapy.[97] In one study, investigators gave 61 healthy older adults (aged 50 to 80 years) either 2 tablespoons of lecithin or a placebo for 5 weeks.[98] Before and at the end of the study they assessed subjects' memory through a brief, standard test and had them keep diaries of memory lapses. By the end of the study, memory test scores of the lecithin group improved significantly and exceeded those of the placebo group. The lecithin group also reported a significant decrease in memory lapses, from about 35 per week before the study to 19 per week at the end of study (a 48% reduction). A larger follow-up study on 117 subjects ages 35 to 80 confirmed these findings.[99] The second study further showed the youngest participants (ages 35 to 49) improved the most, but all age groups had significantly fewer memory lapses.

A chronic, inadequate intake of choline may be involved in the development of Alzheimer's disease (AD). The etiology of AD remains unknown but appears to involve abnormal processing of amyloid precursor protein (APP), a normal brain neuron constituent. APP is a long protein molecule that extends from the cell interior through the membrane to the outside of the cell. APP is degraded by the enzyme secretase on the outside surface of the cell, "clipping" off the outer portion and leaving the rest of the fragment lodged in the cell membrane and poking into the interior. The β-amyloid portion of APP, which is found in the plaque deposits characteristic of AD, is destroyed during the cleavage by secretase. Alternatively, APP may be pulled into the cell and degraded by lysosomal enzymes, generating β-amyloid fragments.[100–102] Although both pathways appear to operate in normal brain tissue, the lysosomal pathway appears to be favored in AD.[103,104]

Evidence suggests the secretase pathway requires PKC for stimulation.[105–107] Thus, reduced PKC levels or activity, possibly due to chronic inadequate choline intake, would be expected to result in increased lysosomal processing of APP and β-amyloid formation. At autopsy, PKC levels have been shown to be reduced in the brains of deceased AD patients compared to normal individuals.[108] The reduced PKC levels may also relate to a second line of evidence concerning the "autocannibalism" of lecithin in nerve membranes.

AD mostly affects cholinergic neurons. These neurons are unique in that they use choline to synthesize the neurotransmitter acetylcholine, phospholipid second messengers, and membrane lecithin.[109,110] These cells obtain most of their choline from the bloodstream or autocannibalism of choline phospholipids in their own membranes. Little choline is derived from phosphatidylcholine synthesized by methylation of phosphatidylethanolamine (see Figure 26.3).

Choline from the blood diffuses bidirectionally across the blood–brain barrier. The net flux is inward toward the brain when blood levels are greater than 14 μ*M* and outward when blood levels are lower.[111] Levels in fasting humans are 8 to 12 μ*M*.[38] Thus, under fasting conditions, brain neurons derive choline largely from autocannibalism of membrane phospholipids. Consumption of choline-rich foods can raise plasma choline levels to 30 μ*M* or higher, resulting in an increase in brain choline and acetylcholine levels.[112]

Potentially at least, prolonged choline insufficiency could lead to continued autocannibalization, membrane disruption, and cell death. The reduced levels of lecithin would also decrease PKC activity and increase lysosomal processing of APP, resulting in more β-amyloid production. The disruption of the membrane may further promote lysosomal processing of APP.[103]

Although some studies of choline or lecithin supplementation in AD patients have suggested possible benefits,[113] most have had disappointing results.[114] It may be that by the time clinical signs of AD appear, it is too late for choline to have much effect. On the other hand, these compounds have been helpful in treating tardive dyskinesia, a disorder involving impaired movement and defective cholinergic nerve transmission.[115]

A variety of defects that result in AD may account for mixed results of choline or lecithin therapy in AD patients.[5,34] Indeed, although the evidence related to APP processing and autocannibalism is intriguing, it is possible they represent a result, rather than the cause of the underlying

defect in AD. Further research is needed to confirm a link among the decline in neuronal membrane phospholipid levels, membrane function, alterations in PKC, APP processing, and amyloid formation, and the expression of AD symptoms.

X. CHOLINE AND PHYSICAL PERFORMANCE

Since cholinergic nerves also carry signals to muscle fibers, it is not surprising that studies also show intense exercise of long duration can lower plasma choline levels and that lecithin and choline supplements prevent this decline and, in many cases, enhance physical performance.

Plasma levels of choline have been shown to drop as a result of intense physical activity of long duration. For example, runners in the Boston Marathon had about a 40% drop in plasma choline levels after the race.[116] Significant drops have also been shown in runners at 20 miles,[117] swimmers,[118] and triathletes.[119]

Studies also show choline supplementation prior to activity increases plasma choline levels and prevents the decline.[120] Moreover, supplementation improves performance in certain activities. In one double-blind crossover study, long-distance runners improved their times in a 20-mile race from an average 158.9 min after taking a placebo to 153.7 min after taking 2.8 g of choline chloride.[117] When one considers that races can be won or lost by seconds, 5 min is a large improvement. Choline also improved swimmers' race times[118] and decreased feelings of fatigue and increased feelings of vigor in swimmers[118] and college basketball players.[121]

Other activities, such as stationary bicycling and running fewer than 20 miles, have not shown improvement with choline supplementation.[120,122,123] However, these studies also showed little or no decline in plasma choline levels during the activity. Thus, their findings are consistent with the hypothesis that when an intense activity reduces plasma choline levels, choline or lecithin supplementation can prevent that drop and improve performance. The findings on choline enhancing both physical and cognitive performance prompted the Committee on Military Nutrition Research of the Food and Nutrition Board to recommend further research on choline.[124]

XI. DRUG INTERACTIONS WITH LECITHIN AND CHOLINE

As noted earlier, methotrexate therapy can reduce liver levels of choline and increase hepatic fatty infiltration; this can be reversed with choline supplementation. Other research on lecithin drug interactions has focused on its ability to protect against gastrointestinal (GI) injury due to nonsteroidal anti-inflammatory drugs (NSAIDs). While NSAIDs are among the most frequently used drugs for pain and fever, they have a well-established ability to cause GI mucosal lesions, perforations, and bleeding, resulting in significant morbidity and even mortality.[125] Enteric-coated NSAIDs have had only limited success due to the delay in therapeutic efficacy. One study showed no difference in the risk of upper GI bleeding from low-dose plain, enteric-coated, or buffered aspirin.[126]

It is thought these adverse effects are due, at least in part, to the association of the NSAID with phospholipids on the extracellular surface of mucosal cells.[127] Normally, these zwitterionic phospholipids maintain the mucosal cell surface in a hydrophobic, nonwettable state, thus protecting it from the corrosive action of stomach acid. When the NSAIDs associate with these phospholipids, the mucosal surface becomes wettable and vulnerable to stomach acid, leading to irritation.

In a rat study, preassociating NSAIDs (aspirin, salicylate, naproxen, diclofenac, and indomethacin) with exogenous phospholipids prevented GI irritation while enhancing the drugs' lipid permeability and therapeutic activity.[127] The reduction in irritation was confirmed in another rat study that also included phenylbutazone, piroxicam, and sudoxicam.[128]

A study in 20 patients with stomach ache due to NSAIDs, steroids, or nonspecific causes showed that soy phosphatidylcholine (2700 mg/day) relieved the pain in 15 patients, reduced it in 3, and had no effect in 2. Overall, there was about a 90% reduction in subjects' self-reported feelings of pain after 8 days of phosphatidylcholine therapy.[128]

The zwitterionic properties of lecithin and other phospholipids also enhance the absorption of various drugs, as reported in the above study of NSAIDs in rats.[127] Other rat studies show that free and lecithin-associated aspirin appear to be equally absorbed into the bloodstream but the latter form has greater antipyretic, anti-inflammatory, and analgesic efficacy. The improved efficacy may be due to increased uptake by target cells, increased binding to cyclooxygenase (the rate-limiting enzyme in the synthesis of proinflammatory and other prostaglandins), or prolonged half-life.[129] Simultaneous administration of antisecretory agents (e.g., omeprazole, ranitidine, and cimetidine) is also advocated as a way to reduce the adverse GI effects of aspirin. Unfortunately, another animal study showed that these drugs increased stomach pH and decreased the absorption of aspirin. However, preassociating aspirin with lecithin corrected this problem.[130]

Lecithin liposomes have been studied in the delivery of macrophage activators in patients with cancer,[131,132] of other drugs administered orally and transmucosally, and of gene therapy.[12]

XII. CHOLINE: AN ESSENTIAL NUTRIENT

Nutritionists define an "essential nutrient" as a substance required but not made in adequate amounts by the organism; therefore, it must be obtained from the diet. Proof a nutrient is essential usually involves showing a deficiency disease when the nutrient is withheld from the diet. In some cases, the nutrient in question may be synthesized by the organism, but not in sufficient amounts. For example, humans can make some niacin (from the amino acid tryptophan), but not enough to prevent pellagra. Therefore, niacin is an essential nutrient for humans and must be obtained from the diet.

Several lines of evidence suggest choline is essential for humans. Human cells grown in culture require choline; deficiency results in severe liver dysfunction in many animal species, including nonhuman primates; humans maintained on choline-deficient TPN solutions frequently develop fatty liver and liver cell damage; and healthy humans fed choline-deficient diets have reduced plasma choline levels and exhibit abnormal liver function within a few weeks.[5] Further, growing evidence shows choline and lecithin play roles cardiovascular health, reproduction and development, learning and memory, and physical performance. As in the case of niacin, we can synthesize choline but probably not enough for our needs. Therefore, we must obtain it from the diet.

Until recently, choline was not regarded as essential for humans by the Food and Nutrition Board, part of the National Academy of Sciences that has established and revised the RDAs since 1941. The RDAs are defined as "the levels of intake of essential nutrients that, on the basis of scientific knowledge, are judged by the Food and Nutrition Board to be adequate to meet the known nutrient needs of practically all healthy persons."[133]

In the last few years, however, the Board has reassessed and expanded the RDAs, replacing them with Dietary Reference Intakes (DRIs). This umbrella concept includes four values (Figure 26.4):[1]

- Estimated Average Requirement (EAR): A nutrient intake value estimated to meet the requirements of 50% of healthy individuals in a group.
- Recommended Dietary Allowance (RDA): The average daily intake level sufficient to meet the nutrient requirement of nearly all (97 to 98%) healthy individuals in a group. An RDA is the EAR plus two standard deviations.
- The Adequate Intake (AI): A value based on observed or experimentally determined estimates of nutrient intake by a group of healthy people — when an RDA cannot be determined.
- Tolerable Upper Intake Level (UL): The highest level of a daily nutrient intake that is likely to pose no risks of adverse health effects to almost all individuals in the general population.

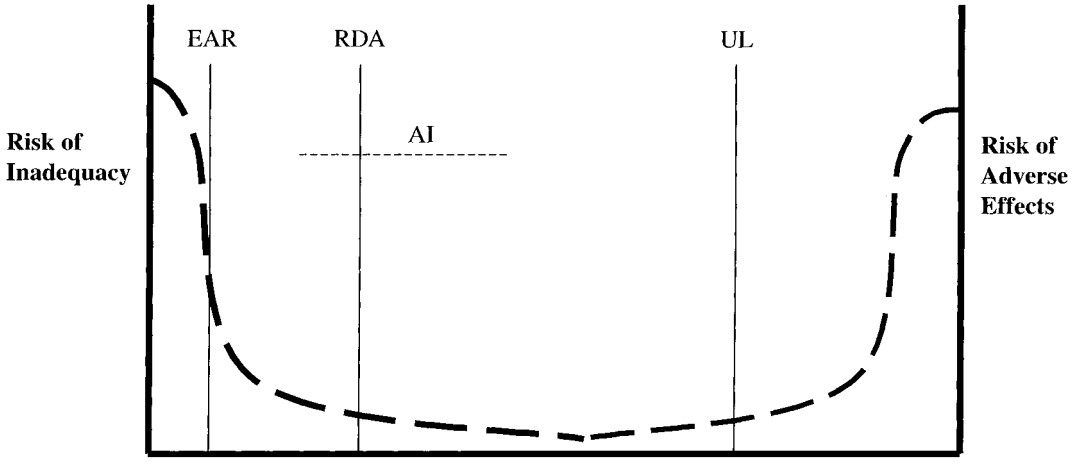

FIGURE 26.4 DRIs.[1] *Abbreviations:* EAR, Estimated Average Requirement; AI, Adequate Intake; RDA, Recommended Dietary Allowance; UL, Tolerable Upper Intake Level.

In April of 1998, the Food and Nutrition Board released its report on DRIs for B-vitamins and choline.[1] While the Board recognized the various lines of evidence supporting choline as an essential nutrient, insufficient evidence was available to determine an EAR (and hence, an RDA). Therefore, the Board recommended AI levels for choline, ranging from 125 mg/day for infants to 550 mg/day for men (Table 26.4).

XIII. SUMMARY AND RECOMMENDATIONS FOR RESEARCH

Choline appears to be an essential nutrient for humans. It is required for normal liver function and, as a component of such substances as lecithin, sphingomyelin, platelet-activating factor, and acetylcholine, may play roles in cardiovascular health, reproduction and development, and,

TABLE 26.4
DRIs for Choline (mg/day)[1]

Population Group	Adequate Intake	Tolerable Upper Limit
	Infants	
0–5 months	125	Not determined
6–11 months	150	Not determined
	Children	
1–3 years	200	1000
4–8 years	250	1000
9–13 years	375	2000
	Males	
14–18 years	550	3000
19+ years	550	3500
	Females	
14–18 years	400	3000
19+ years	425	3500
Pregnant (all ages)	450	3500
Lactating (all ages)	550	3500

perhaps, memory and physical performance. The metabolism of choline is interrelated with that of folate, methionine, and homocysteine. Lecithin and other choline phospholipids are involved in cell signaling; defects in this process may be involved in the development of AD and certain types of cancer.

Dietary intake of choline in the United States has probably declined over the last 25 years because of a decreased intake of foods high in fat and cholesterol, which are major sources of choline and lecithin. Some have speculated that current intakes among some segments of the population are suboptimal.

Further research on the roles of lecithin and choline in health and disease is needed in such areas as:

- Comprehensive analysis of choline and choline phospholipid content of foods and dietary intake levels.
- Examination of graded levels of choline intake and how they relate to various parameters of health, such as tissue and serum levels of choline compounds, homocysteine, cholesterol, and folate; and to the functioning of various organs.
- The interrelationships among choline, folate, vitamin B_6, vitamin B_{12}, and methionine, and their relative contributions to homocysteine metabolism.
- How choline phospholipids participate in cell signaling, how they regulate the diverse functions that involve this central process, and how perturbations in cell signaling lead to various disease states.

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27 Conjugated Linoleic Acid: The Present State of Knowledge

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I. INTRODUCTION

Conjugated linoleic acid (CLA) is the name given to describe a group of positional and geometric isomers of octadecadienoic acid (18:2). The double bonds in CLA are conjugated and not separated by a methylene group ($-CH_2-$) as in linoleic acid (LA, 18:2n-6), an essential fatty acid. Furthermore, CLA will not substitute for LA as an essential fatty acid. The isomers of CLA occur naturally in food products derived from ruminant sources (beef, lamb, and dairy) because of the process of bacterial biohydrogenation of LA, which is present in feedstuffs for these animals, in the rumen.[1–3] CLA is also found naturally in a wide variety of food products other than ruminant origin, including seafood, turkey, and vegetable oils.[2]

Fats and meats from ruminant species are the richest natural source of CLA isomers. These isomers are found to be in milligrams per gram of fat in the meats from lamb, veal calves, and cattle.[4] The highest concentrations of CLA in food are present in various dairy products.[5,6] In most cases, the *cis*-9, *trans*-11 isomer, which recently was proposed to be named rumenic acid,[7] is the predominant isomer of CLA found in food except for vegetable oils that can contain several other

0-8493-8734-5/01/$0.00+$.50
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isomers. Estimates of CLA intake range from 0.3 to 1.5 g/person/day and appear to be dependent on gender and the intake of foods from animal and vegetable origin.[5]

The presence of CLA in dairy fats has been known for years. The discovery that CLA isolated from grilled beef inhibited chemically induced cancer[8–10] is what prompted the attention of the scientific community to study its physical and biological properties. The growing body of literature on CLA suggests that these isomeric conjugated fatty acids possess potent biochemical and physiological activities that could benefit human health and protect against chronic disease.

II. CHEMISTRY AND NOMENCLATURE

The acronym CLA is used to describe a family of octadecadienoic acid (18:2) isomers that possess a pair of conjugated double bonds along the alkyl chain. Theoretically, the double bonds could exist at any location from Δ2 to Δ18 carbons to yield numerous possible isomeric structures. Isomeric forms of octadecadienoic acid have been reported to contain conjugated double bonds at positions 7,9; 8,10; 9,11; 10,12; 11,13; and 12,14 (counting from the carboxyl end of the molecule) in chemically prepared CLA mixtures or natural products.[11–14] Each of the aforementioned positional conjugated diene isomers can occur in one or more of the following four geometric configurations: *cis-,trans-*; *trans-,cis-*; *cis-,cis-*; or *trans-,trans-*, which would give 24 possible isomers of CLA.[11] Most of these isomers are contained in commercially available preparations of CLA supplements produced under alkaline conditions from vegetable oils (sunflower and safflower) containing a high concentration of LA.[15]

The most common CLA isomer found in meat from ruminant species and bovine dairy food products is octadeca-*c*9,*t*11-dienoic acid.[16] The presence of minor components, such as the *t*7,*c*9, *t*8,*c*10, *t*10,*c*12, *t*11,*c*13, *c*11,*t*13, and *t*12,*t*14 isomers, and their *cis,cis*, *trans,trans* isomers were also reported in natural products.[12,13] Two trivial names have been proposed for the *c*9,*t*11 isomer, bovinic acid[17] and rumenic acid.[7] The name bovinic acid is considered to be too restrictive for CLA because the *c*9,*t*11 isomer is not only produced in the rumen of the bovine but also produced by other ruminant animals by the same mechanism. The name rumenic acid is now gaining acceptance, evidenced by its repeated appearance in the literature.

Naturally occurring CLA in ruminant meat and dairy products is believed to be formed by bacterial isomerization of LA and possibly α-linolenic acid (18:3n-3) from grains and forages to the *c*9,*t*11–18:2 in the rumen of these animals.[1,2,18] CLA may also be formed during cooking and processing of foods. For example, Ha and colleagues[18] measured the CLA content in processed cheeses and proposed that the formation of CLA is from a free radical–type oxidation of LA that could be affected by aging, heat treatment, and protein quality. According to this hypothesis, allyl radicals are produced from the oxidation of LA in glycerol lipids including phospholipids during heat treatment under anaerobic conditions. CLA is derived after the allyl radicals are stabilized by reprotonation from proteins acting as hydrogen donors.[18]

III. ANALYSIS OF CLA IN FOOD AND BIOLOGICAL SAMPLES

The analysis of CLA in food and other biological samples follows the general guidelines for the determination of fatty acid composition with particular emphasis on the derivatization methods. The conjugated double bonds in CLA are less stable than the methylene interrupted double bonds as in LA. It is reported that the stability of CLA is similar to that of arachidonic acid (20:4n-6) and docosahexaenoic acid (DHA, 22:6n-3).[19,20] Therefore, preventing oxidation of these labile fatty acids is critical for a successful analysis, and more caution should be taken in dealing with samples for CLA determination. Three basic steps are required to complete a routine lipid analysis of fatty acids: lipid extraction, fatty acid derivatization, and analytical determination. For samples like oils and fats from plant and animal sources, lipid extraction is not necessary. In some cases, extra steps are taken to purify and/or fractionate the extracted lipids into several classes, such

as triacylglycerols, free fatty acids, and phospholipids to obtain more precise and detailed fatty acid information. Although the extracted lipid could be used for direct analysis of its composition, such as in the analysis of triacylglycerol, most often, the extracted lipids are subjected to a chemical process to derivatize the fatty acids. The fatty acid products can then be quantified by chromatography. Two common instruments used are the gas chromatograph (GC) and high-performance liquid chromatograph (HPLC).

The most commonly used derivative for fatty acid analysis is the fatty acid methyl ester (FAME). Numerous methods are available for the preparation of FAME, but only a few are suitable in the analysis involving CLA. The fatty acid derivatization is a critical part of any fatty acid analysis. Therefore, the emphasis of the current section will be on the comparison of different methylation methods and the recommendations for the commonly accepted application.

Various methods are currently available for the preparation of FAME. Both acid and alkaline catalysts can be used for this purpose. The acid catalyzed methods include BF_3 (10 to 14%, w/v) in methanol, BCl_3 (10%, w/v) in methanol, anhydrous HCl (5%, w/v) in methanol, and concentrated sulfuric acid (1 to 2%, v/v) in methanol. Two base-catalyzed methods are 0.5 *N* sodium methoxide and tetramethylguanidine (TMG) in methanol (1:4, v/v).

There are advantages and limitations for each of the aforementioned reagents. The BF_3 method, since its introduction by Metcalfe and colleagues[21,22] has been widely used and is adopted as the official methods for both AOCS (method Ce 2–66 and Ce 1b-89) [23] and AOAC (method 969.33).[24] The advantage of the BF_3 method is that it is very fast to methylate free fatty acids and if combined with a saponification step, as in the official methods, it is also very fast in methylating esterified fatty acids. On the other hand, BF_3, as well as other acid catalysts, will change the double-bond configuration of fatty acids such as those in conjugated dienes (shift of the *cis-,trans-/trans-,cis-* configuration to a *trans-trans-* configuration),[25,26] produce methoxy artifacts,[25,27] and form artifact CLA isomers from hydroxy fatty acids if present in the lipid sample.[27]

The alkaline catalysts perform better on lipids containing fatty acids with unique conjugated diene structures. Isomerization and artifacts are not produced when sodium methoxide or TMG are used as transesterification agents; however, they do not methylate free fatty acids and *N*-linked (amide-bond) fatty acids such as those found in sphingolipids. Although the original author on the TMG method claimed that it worked fine with free fatty acids,[28] recent tests by our laboratory and other researchers showed different results. Those reports conclude that TMG is not effective in methylating free fatty acids and phospholipids.[25,26] Therefore, these methods are not suitable for samples with high acid values (high free fatty acid content). Kramer and colleagues[25] reported that CLA analysis using both acid and alkaline catalysts in sequence gave improved results, that is, less CLA loss with acid catalyzed methylation, but not as much with base catalyzed. There is no single method that works optimally in all situations. Researchers need to know the nature of the sample that they are dealing with and to select a method accordingly.

As research continues on the actions of CLA, it is essential to provide accurate and precise compositional analyses in food and biological samples to interpret data from numerous investigations correctly. Therefore, we include a basic protocol using sodium methoxide in methanol as the catalyst for the analysis of CLA. We have used this method on a variety of food and biological samples. The sample is extracted, lipid isolated (up to 50 mg of lipid), and FAME prepared using sodium methoxide according to the method used by Li and Watkins.[29] Specifically, the lipid is dissolved in dry toluene (1 ml) in a test tube with a Teflon-lined screw cap, 2 ml of 0.5 *M* sodium methoxide in anhydrous methanol added, and the tube placed in a heating block at 50°C for 10 min. Next, 0.1 ml of glacial acetic acid is added to stop the reaction followed by the addition of 5 ml of deionized water and 3 ml of hexane to the tube. The tube is placed in a shaker for 10 min and then centrifuged at 1000 *g* for 10 min to separate the hexane and the aqueous layers. The upper hexane layer is transferred to another tube containing 1 mm dried sodium sulfate and the aqueous phase is extracted again with 3 ml of hexane and the hexane layers combined. The sample containing FAME is dried under a gentle nitrogen stream and

reconstituted with isooctane. The actual volume of hexane may vary depending upon the amount of lipid extracted and the gas chromatographic procedure.

Various instruments are adopted to separate and characterize the CLA isomers. A GC equipped with a polar 30 to 100 m capillary column and a flame ionization detector (FID) is the most widely used instrument to characterize FAME including those derived from CLA in a given lipid sample. Mossoba and colleagues[30] reported that ten CLA peaks were resolved in a commercial CLA preparation by GC equipped with a 100 m column (SP 2560, Supelco, Inc., Bellefonte, PA or CP-Sil 88, Chrompack, Bridgewater, NJ). In the aforementioned procedure the ten peaks resolved may actually represent more than 15 CLA isomers since the method and column have a limited capacity to separate fully all geometric and positional isomers. Generally, it is not possible to identify every individual CLA isomer by conventional capillary GC analysis. A more powerful approach has been the application of argentation (silver ion) HPLC (Ag^+-HPLC) equipped with an ultraviolet (UV) detector and a wavelength of 233 nm. Sehat and colleagues[11] has described such a method that resolves CLA isomers according to geometric configuration and position of the conjugated diene structure. Moreover, Mossoba and colleagues[31] reported that 16 isomers of CLA were resolved by an Ag^+-HPLC method. Although the Ag^+-HPLC method is more powerful than GC in resolving CLA isomers, it cannot be used to quantify other fatty acids with the UV detector because only CLA molecules are absorbed at 233 nm. Therefore, ideally if the two methods were combined, using GC for the general fatty acid analysis and Ag^+-HPLC for the CLA analysis, the results of these two procedures would provide a more complete profile of FAME derived from food and biological samples.

Other techniques can be used to determine the structural configuration of unknown CLA isomers that can be less time-consuming than combining the analyses from GC and HPLC. To obtain more specific detail of CLA isomers, the following methods can be used: GC-electron ionization mass spectrometry (GC-EIMS), GC-direct deposition-Fourier transform infrared spectroscopy (GC-DD-FTIR), nuclear magnetic resonance spectroscopy (NMR), and ^{13}C NMR. Detailed information on the application of these advanced analytical methods for CLA analysis is beyond the scope of this discussion. Interested readers can refer to a recently published book on CLA for additional information.[32]

IV. CLA CONTENT IN FOOD PRODUCTS AND BIOLOGICAL SAMPLES

Among all food products, ruminant meat and dairy products (milk, butter, cheese, and yogurt) have been found to contain the greatest amounts of CLA.[2,18,33–35] Fats and meats from ruminant species are the richest natural source of CLA and the reported values ranged from 2.7 to 5.6 mg of CLA/g fat in lamb, veal, and beef. The CLA content can be much higher (0.5 to 12 mg CLA/g fat) in dairy products.[2,5,6,36,37] In most cases, the *c*9,*t*11 isomer or rumenic acid,[7] is the major isomer of CLA found naturally except for plant oils that can contain several other isomers. The reported CLA content in commercial and natural food products is summarized in Table 27.1.

Dairy products, especially cheeses, are a chief source of dietary CLA in animal-derived foods. The CLA concentrations in various dairy products (cheeses, milk, butter, buttermilk, sour cream, ice cream, and yogurt) range from 0.55 to 24 mg/g fat (Table 27.1). The average CLA content in milk is about 10 mg/g milk fat.[5,37] The largest variation in the amount of CLA is found in various natural cheeses that range from 0.55 to 24 mg/g fat (Table 27.1). Seven CLA peaks that could represent nine isomers were present in dairy products; among these, *c*9,*t*11, *t*10,*c*12, *t*9,*t*11, and *t*10,*t*12 accounted for more than 89%.[18] The CLA content in cheeses is primarily determined by the CLA content in the milk, which varies in CLA concentration due to seasonal variation, geography, nutrition of the cow, and management practices. In addition, CLA content of cheese is also affected to some extent by the production process and maturation.[38]

Reported values for CLA content in beef muscle vary considerably from 1.2 to 9.9 mg/g fat.[2,18,39,40] Fritsche and Fritsche[41] reported that the amount of the *c*9,*t*11 18:2 isomer in beef

TABLE 27.1
CLA Content and Isomeric Distribution in Commercial and Natural Food Products

Products	CLA Content (mg/g fat)[a]	CLA Isomer Distribution[b]	*c*9,*t*11-18:2, %	Ref.
		Natural Cheeses		
Blue	0.55–7.96	1/2, 4, 5, 8, 9, 10, 12/14	15–100	2, 18, 62, 140
Brie	3.8–4.75	1	100	40, 140
Brick	7.1	1	91	2
Cheddar	1.36–5.86	1/2, 4, 5, 8, 9, 10, 12/14	18–100	2, 18, 33, 34, 40, 62, 140, 142, 151
Colby	6.1	1	92	2
Comte	15.0–20.8	1/3, 2/4, 5, 7/8, 9/10, 11, 12/13/14		38
Cottage	4.5–5.9	1	83–100	2, 40
Cougar Gold	3.20–5.17	1/2, 4, 5, 8, 10, 12/14	85–100	140, 142, 151
Cream	0.94–4.30	1/2, 4, 5, 8, 9, 10, 12/14	88–100	2, 18, 40, 140
Edam	5.38	1	100	140
Farmer cheese	4.7	1	100	40
Gouda	5.96	1	87	34
Monterey Jack	4.80	1	100	140
Mozzarella	4.31–4.96	1	84–100	2, 34, 40, 140
Muenstre	6.6	1	93	2
Parmesan	1.93–8.6	1/2, 4, 5, 8, 9, 10, 12/14	38–100	2, 18, 40, 140, 143
Pecorino	13.0	1		143
Ricotta	5.6–24.2	1	84	2, 143
Romano	1.11–2.9	1/2, 4, 5, 8, 9, 10, 12/14	16–92	2, 18
Swiss	5.45–14.2	1	90–100	2, 140, 143
Viking	3.13–5.39	1/2, 4, 5, 8, 10, 12/14	87–100	140, 142, 151
		Processed Cheeses		
Processed	1.81–6.2	1/2, 4, 5, 8, 9, 10, 12/14	18–100	2, 18, 40, 140
Velveeta	5.17–5.2	1	48–86	2, 33
Spread	1.0–4.5	1/2, 4, 5, 8, 9, 10, 12/14	16–100	2, 18, 140
Cheese Whiz	4.9–8.81	1/2, 4, 5, 8, 9, 10, 12/14	21–100	2, 18, 33, 40
Kroger Squeeze cheese	8.92	1	68	33
Kroger Nice and Cheesy	6.00	1	63	33
Kraft American singles	3.19	1	58	33
Goat cheese	2.7	1	100	40
		Butter		
Butter	4.7–8.11	1	78–90	2, 34, 40, 62
		Fermented Dairy Products		
Buttermilk	4.66–5.4	1	89–100	2, 140
Sour cream	4.14–7.49	1	78–100	2, 34, 40, 62, 140
Yogurt	1.7–9.01	1	71–100	2, 34, 40, 62, 140, 143
		Ice Cream		
Ice cream	3.6–4.95	1	76– 86	2, 34
		Milks		
Cow milk	0.7–10.1	1/2, 4, 5, 6, 8, 9, 10, 12/14	59–100	2, 18, 40, 60, 62, 140, 141, 143, 144

continued

TABLE 27.1 (CONTINUED)
CLA Content and Isomeric Distribution in Commercial and Natural Food Products

Products	CLA Content (mg/g fat)[a]	CLA Isomer Distribution[b]	*c*9,*t*11-18:2, %	Ref.
		Natural Cheeses		
Sheep milk	10.8–29.7	1, 5, 6, other c/t, t/t	99–100	141, 143, 144
Goat milk	6.1–10.35	1, 5, 12	99–100	67, 144
Mare	0.9	1, 5, 12	99–100	144
Sow	2.2	1, 5, 12	99–100	144
Human milk	1.7–36.4	1, 5, 12	83–100	17, 48, 49, 50, 144
		Meats and Poultry Products		
Beef products, uncooked	1.2–8.5	1/2, 4, 5, 8, 9, 10, 12/14	21–61	2, 18, 39, 40
Cooked beef	3.3–9.9	1/2, 4, 5, 8, 9, 10, 12/14	19–84	2, 18, 39, 40
Beef tallow	2.6	1	84	2
Lamb	5.6	1	92	2
Pork	0.6	1	82	2
Chicken	0.9	1	84	2
Turkey	2.5	1	76	2
Smoked bacon	0.8–2.6	1	75–78	2
Turkey frank	1.6	1	70	2
Smoked turkey	2.4	1	62	2
		Plant Oils		
Plant oils[c]	0.1–0.7	1, 5	38–47	2
		Egg Yolk		
Egg yolk	0.0–0.6	1	82	2, 42, 43, 44
		Seafood		
Seafood[d]	0.3–0.6	n.a.e	n.d.[e]	2

[a] Some values were originally expressed as percentage of total FAME or mg/g fatty acids. These values are converted to mg/g fat by using a multiplication factor of 9.5 or 0.95, respectively.[49]

[b] Each number represents a CLA isomer designated as follows: 1 = *c*9,*t*11; 2 = *t*9,*c*11; 3 = *c*8,*t*10, 4 = *c*10,*t*12, 5 = *t*10,*c*12; 6 = *t*7,*c*9; 7 = *c*8,*c*10; 8 = *c*9,*c*11; 9 = *c*10,*c*12; 10 = *c*11,*c*13; 11 = *t*11,*t*13, 12 = *t*9,*t*11; 13 = *t*8,*t*10; 14 = *t*10,*t*12. Isomers separated by "/" co-elute during chromatographic analysis either by GC or HPLC.

[c] Safflower oil, sunflower oil, peanut oil, canola oil, vegetable oil, corn oil, coconut oil, and olive oil.

[d] Including salmon, lake trout, sea scallops, shrimp, and mussels.

[e] n.d., not detected; n.a., not available.

averaged 0.76% of total FAME for fat samples from bulls and 0.86% for fat from steers. Minor isomers, e.g., *t*9,*c*11, *c*9,*c*11, and *t*9,*t*11, were also found in beef fat samples. Others have reported that the *c*9,*t*11 18:2 content in beef ranged from 1.7 to 6.5 mg/g fat[39] and 0.65% of total FAME in beef fillet.[5]

CLA is also present in small amounts in food products not originating from ruminant animals. Turkey meat has the highest CLA content of 2.5 mg/g fat for nonruminant species.[18] Chicken also contained CLA (0.9 mg/g fat) as did pork (0.6 mg/g fat) with *c*9,*t*11 being the major isomer of CLA (84 and 82%, respectively).[2] The amount of CLA in chicken egg yolk lipids ranges from 0 to 0.6 mg/g fat.[2,42–45] CLA is also present in plant oils (0.1 to 0.7 mg/g fat) and selected seafood (0.3 to 0.6 mg/g fat) in small quantities.[2] Unlike in ruminant-derived foods where the *c*9,*t*11 is the chief isomer of CLA, this isomer accounts for only 38 to 47% of the total CLA in plant oils and, rather interestingly, appears to be absent in seafood lipids.

Although CLA is found almost exclusively in animal products, plant oils (safflower oil, sunflower oil, peanut oil, canola oil, vegetable oil, corn oil, coconut oil, and olive oil) contained very low levels ranging from 0.1 mg (coconut oil) to 0.7 mg/g oil (safflower oil).[2] Among the isomers found in these plant oils, the *c*9,*t*11 18:2 accounted for 37 to 44% of the total CLA. Banni and colleagues[46] carried out a series of analyses to characterize the fatty acids with conjugated dienes in partially hydrogenated oil (mixture of partially hydrogenated soybean oil and palm oil) and confirmed the presence of CLA isomers in partially hydrogenated oils. Moreover, Mossoba and investigators[30] reported that conjugated *cis,trans* and *trans,trans* 18:2 isomers of linoleic acid were present in hydrogenated soybean oil and margarine.

V. CLA IN HUMAN TISSUES

CLA has been identified in various human tissues, such as adipose and serum, and in bile, breast milk, and duodenal secretions.[47] Fogerty and colleagues[48] reported 5.8 mg/g fat of CLA in human milk for subjects consuming a normal Australian diet. Precht and Molkentin[49] reported a value of 3.8 mg/g fat (range of 2.2 to 6.0 mg/g fat) in human milk obtained from 40 German women. McGuire and colleagues[17] analyzed 14 human milk samples from subjects in the Pacific Northwest and reported that CLA values ranged from 2.23 to 5.43 mg/g fat (mean 3.81 mg/g fat) or 0.02 to 0.30 mg/g on a milk weight basis. In the study, all milk samples contained from 83 to 100% of the *c*9,*t*11 isomer and in 8 out of the 14 samples, the *c*9,*t*11 isomer was the only form of CLA in milk. Jensen and colleagues[50] reported a lower level of CLA in human milk that ranged from 1.4 to 2.8 mg/g fat with an average of 1.8 mg/g fat. Based on these data, CLA in human milk for Western societies ranged from 1.4 to 5.8 mg/g fat.

The concentration of CLA found in human plasma and serum appears to be linked to dietary fat type and food consumption patterns. Herbel and colleagues[47] reported that plasma CLA concentration ranged from 6.4 to 7.3 μmol/l in a human study in which subjects were given a high level of safflower oil for 6 weeks. The high LA intake from safflower oil did not increase the plasma level of CLA indicating that dietary LA is not converted to CLA in these subjects. In another study, the effect of *trans* fatty acid intake on serum CLA was studied.[51] In the study, 80 human subjects were put on a diet high in saturated fatty acids mainly from dairy fat for 5 weeks; then they were separated into two dietary groups, 40 subjects were assigned on a diet high in *trans* fatty acids from partially hydrogenated vegetable oil, and the other 40 were on a similar diet high in stearic acid. At the termination of the study, serum samples from the *trans* diet group (CLA was 0.43% of total fatty acid or 4.1 mg/g fat) contained 30% more CLA than samples from the dairy fat diet (0.32% CLA or 3.0 mg/g fat). Samples from those given the stearic acid diet had only half of the amount of CLA compared with those given the dairy fat diet. These data indicate that a possible relationship exists between *trans* fatty acid intake and its effect on elevating serum CLA concentration. In another dietary intervention study of nine healthy men, cheddar cheese was added to their diet at a level of 112 g/day for 4 weeks. Plasma CLA concentration increased from 7.1 to 9.6 μmol/l at the end of the supplement period and maintained at 7.8 μmol/l after another 4 weeks following intervention.[52]

The concentration of and isomeric distribution for CLA have recently been characterized in human adipose tissue. Fritsche and colleagues[53] reported that human subcutaneous adipose tissue contained two major CLA isomers, both *c*9,*t*11 and *t*9,*t*11, and two minor isomers *t*9,*c*11 and *c*9,*c*11.

The presence of CLA in human tissues is principally due to dietary intake since the amount of *c*9,*t*11 18:2 in human adipose tissue appears to be directly related to milk fat intake.[54] However, the possibility of endogenously produced CLA in human tissues could not be excluded. Brown and Moore[55] have reported the presence of CLA producing bacterial strains of *Butyrivibrio fibrisolvens* isolated from human feces. Moreover, CLA is also produced in conventional, but not germ-free rats after consumption of LA.[56] On the other hand, consuming safflower oil that is high in triacylglycerol-esterified LA, the precursor for bioisomerization, did not increase the CLA concentrations

in total lipids of human plasma.[47] Therefore, the contribution of colon-derived CLA to the total amount of CLA in human tissues and in nonruminant monogastric species is most likely negligible. Another possible mechanism for the origin of human tissue CLA is by the desaturation of *trans*-vaccenic acid as shown in rat liver microsomal preparations.[57] The results of Salminen and colleagues[51] further supported this mechanism by showing that a high level of dietary *trans* fatty acids actually increased serum CLA content over stearic acid. However, the *trans* fatty acid intake (more than 2 g/day) in this study was three- to tenfold greater than the habitual diet.[58] Further study is required to determine the impact of exogenous amounts of dietary *trans* fatty acids on tissue CLA concentrations in the human.

VI. CREATING DESIGNED FOODS ENRICHED WITH CLA

The current human estimated intake of CLA by dietary sources is not enough to exert the potential beneficial biochemical, molecular, and physiological effects against cancer, atherosclerosis, and obesity based on studies with various animal models. Ip and colleagues[59] estimated, on the basis of a rat model, that a 70 kg human should consume 3.0 g CLA/day to obtain its beneficial effects. The calculation was determined from rats given a diet supplemented with CLA at 0.1% of the total diet and would reflect about a threefold higher intake of 1 g CLA/day by an average person in the United States.[18] Therefore, the potential health-promoting capabilities to be achieved with CLA will not be realized without supplements or changes made in the food supply. Since supplements are less desirable than food as a source of nutrients, nutraceuticals, and phytochemicals, a far better approach would be to increase the content of CLA by creating designed foods enriched with CLA.

Two approaches can be taken to increase the amount of CLA in foods. The first is to consume more CLA-rich foods of ruminant origin. The second is to increase the CLA content in eggs, milk, and meat as animal-derived designed foods. The latter approach is more practical since it would not depend on changing dietary practices or elevating the daily intake of cholesterol and saturated fat.[60] Increasing the CLA content of food products like milk and meat also has the potential of increasing their nutritional and therapeutic value, and could favorably influence marketing of value-added designed foods.

The CLA content in food is affected by many factors during every stage from the field to mouth, including raw material production, processing, packaging, storage, and food preparation before serving. The original CLA content in the raw material is determined at the time of collection or after minimal processing. Although subsequent processing, storage, and food preparation, will modify the CLA content to some extent, this variation is fairly small compared with the large natural variation found especially in dairy products.[26,33,34,60] Therefore, the focus of this section is on approaches that have been tested to enhance the CLA content in various food products, such as milk, meat, eggs, and vegetable oil. Detailed information on CLA enrichment in some of the studies presented here is given in Table 27.2.

Several factors, such as type of feed (nutritional factor), season, genetic variation, and management factors can influence the concentration of CLA in ruminant fats, meat, and dairy products.[60–62] In general, changes in feed, season, and management factors will all lead to the changes in substrate supply and extent of biohydrogenation of LA. This in turn will affect the supply of intermediate and end products of biohydrogenation, thus influencing the CLA content in milk and meat from ruminants.[60,61]

A. Milk and Dairy Products

Nutritional and management factors will influence the CLA content of milk. Jiang and colleagues[37] conducted a feeding study using 28 lactating dairy cows to examine the effects of diets and different dietary regimens on the CLA concentration in milk. During the study, cows were divided into three groups and assigned to two dietary treatments. The control group was fed a diet with an average

TABLE 27.2
CLA Content and Isomeric Distribution in Designed Foods or After Dietary Supplementation

Food Products and Treatment		CLA Content (mg/g fat)[a]	CLA Isomer Distribution[b]	c9,t11–18:2, %	*T*10,c12–18:2, %	Ref.
		Cow milk				
Cows were given dietary treatments as listed at right	Normal diet	3.6–5.0	1	100	0	60
	High oil corn	3.7		100	0	
	1/3 pasture	8.5		100	0	
	2/3 pasture	13.6		100	0	
	Pasture	21.0		100	0	
	3% fish meal	8.2–8.5		100	0	
	High grain or conserved forage	6.9–8.6		100	0	
Treatments were given to cows at 53 g/kg dietary dry matter	Peanut oil	13.3	1	100		61
	Sunflower oil	24.4		100		
	Linseed oil	16.7		100		
		Goat Milk				
Goats were fed canola oil with grain	0 canola	10.35	1/2, 9	n.a.[c]	n.a.	67
	2% (40 g)	19.42		n.a.	n.a.	
	4% (80 g)	32.05		n.a.	n.a.	
	6% (120 g)	29.46		n.a.	n.a.	
		Egg yolk				
CLA source (Pharmanutrients Inc.) contained 65% CLA	2.5% CLA, 1 d	45.7	1, 5	52	48	42
	2.5% CLA, 49 d	45.5		54	46	
	5.0% CLA, 1 d	81.9		50	50	
	5.0% CLA, 49 d	80.8		52	48	

continued

TABLE 27.2 (CONTINUED)
CLA Content and Isomeric Distribution in Designed Foods or After Dietary Supplementation

Food Products and Treatment		CLA Content (mg/g fat)[a]	CLA Isomer Distribution[b]	c9,t11–18:2, %	*T*10,*c*12–18:2, %	Ref.
		Egg yolk				
CLA source: PharmaNutrients, Tonalin, 60% CLA, 37% *c*9,*t*11, 47% *t*10,*c*12, 16% other isomers	0.5% CLA, 11 d	8.0	1, 5	60%	40	43
	2.5% CLA, 11 d	57.7		55%	45	
	5.0% CLA, 11 d	111.1		45%	55	
	0.5% CLA, 26 d	8.3		67%	33	
	2.5% CLA, 26 d	61.3		53%	47	
	5.0% CLA, 26 d	111.6		53%	47	
CLA source: Conlinco, Inc., 62% CLA (17.9% *c*9,*t*11, 20.3% *t*10,*c*12) 2 wk feeding	1.25% CLA	30.5	1, 3, 5, 15	43%	32	44
	2.5% CLA	64.8		40%	37	
	5.0% CLA	236.5		22%	24	
CLA source: PharmaNutrients, Tonalin™, 55–56% CLA in soft-gel capsule	1 g CLA every other day	66.5	1/2, 5, 8, 9	n.a.	n.a.	45
	1 g CLA every fourth day	114.0				
		Fish muscle				
CLA source: Saskatchewan Wheat Pool, 66.9% CLA (24.9% *c*9,*t*11, 35.2% *t*10,*c*12)	0.5% CLA diet	32.3	1, 5, 14	44%	47	79
	0.75% CLA diet	41.8	1, 5, 8, 14	41%	50	
	1.0% CLA diet	77.0		40%	51	
		Other				
Hydrogenated vegetable oil	CLA content increased with the progress of hydrogenation	0.90–98.27	1/3, 4/2, 5, 15 or 16, 7/8, 9/10, 12/13/14		Check ref.	80

[a] Some values were originally expressed as percentage of total FAME or mg/g fatty acids. These values are converted to mg/g fat by using a multiplication factor of 9.5 or 0.95, respectively.[49]

[b] Each number represents a CLA isomer designated as follows: 1 = *c*9,*t*11; 2 = *t*9,*c*11; 3 = *c*8,*t*10, 4 = *c*10,*t*12, 5 = *t*10,*c*12; 6 = *t*7,*c*9; 7 = *c*8,*c*10; 8 = *c*9,*c*11; 9 = *c*10,*c*12; 10 = *c*11,*c*13; 11 = *t*11,*t*13, 12 = *t*9,*t*11; 13 = *t*8,*t*10; 14 = *t*10,*t*12; 15 = *c*11,*t*13; 16 = *t*11,*c*13. Isomers separated by "/" co-elute during chromatographic analysis by GC or HPLC.

[c] n.a., not available.

ratio of forage to concentrate of 50:50 by weight. The two trial groups were fed a test diet having lower forage-to-concentrate ratio offered either as a restricted or nonrestricted intake basis. Variation of CLA in milk was substantial (2.5 to 17.7 mg/g fat). The difference in the CLA content of milk was not a direct effect of dietary ratio of forage to concentrate, but was due to the different feeding regimen between the two treatment groups. Cows on the feed-restricted treatment of the test diet exhibited, on average, the highest concentration of CLA in milk (11.28 mg/g fat). For cows offered the test diet *ad libitum*, the CLA concentration in their milk did not differ significantly from that of cows given the control diet (6.6 vs. 5.0 mg/g fat). The author suggested that the CLA content of milk could be increased through manipulation of diet and management of the cowherd.

A study by Dhiman and colleagues[60] showed that the CLA content in bovine milk increased linearly as the amount of pasture was increased in the ration. Cows grazing pasture alone had 150% and 53% more CLA in milk fat than cows given pasture that consisted of 1/3 and 2/3 of the ration, respectively. A supplement was given to the cows in the 1/3 and 2/3 pasture treatment groups to balance their nutritional needs. Increasing the proportion of grazed grass from pasture in the diet of dairy cows had a positive effect on increasing the CLA content of milk. This study suggested that α-linolenic acid (18:3n-3) might be a substrate for conversion to CLA because it is the predominant unsaturated fatty acid in pasture grass, and it was the pasture-dominated rations that supported the highest CLA content in milk fat.[60] Interestingly, providing fish meal to cows also increased the CLA content of milk by a small margin.[60]

Supplementation of the bovine ration with fats containing LA resulted in increased levels of CLA in milk.[61,63,64] Stanton and colleagues[63] studied the effects of grass dry matter allowance and dietary supplements of full-fat rapeseed on CLA levels in bovine milk. Grass allowance of 16 kg/cow/day resulted in reduced milk fat CLA level (3.91 mg CLA/g fat) compared to 20 kg/cow/day after 19 weeks of treatment. The CLA content increased significantly ($p < 0.001$) in milk fat of cows on a high-rapeseed-supplemented ration (1650 g/cow/day full-fat rapeseed) compared to the control (pasture fed) and low-rapeseed supplemented ration (825 g/cow/day full-fat rapeseed). In another study by the same investigators, cows on pasture were provided full-fat soybean or ground full-fat rapeseed. The CLA content in the milk of cows provided the rapeseed ranged from 10.6 to 33.5 mg/g fat and for soybean supplemented 8.8 to 30.5 mg/g fat. Both treatments resulted in a significantly higher amount of CLA in milk than cows given the control ration (6.8 to 25.7 mg/g fat). Milk yield and milk constituent yields were not affected by supplementation with either full-fat soybean or rapeseed treatments compared with controls, but milk protein concentration was significantly reduced by both oilseed supplements.

Administration of CLA to cows by abomasal infusion resulted in increased concentrations of CLA in milk fat. Chouinard and colleagues[65] reported that cows receiving exogenous CLA infusion produced as much as 63.6 mg CLA/g fat compared with control cows, which had the normal yield of 6.8 mg CLA/g fat. All of the major CLA isomers in the supplement were transferred to the cow's milk in a dose-dependent manner. The exogenous CLA isomers, however, altered milk fatty acid composition and reduced bovine milk fat concentration and yield by as much as 55%, presumably by inhibiting *de novo* fatty acid synthesis in the mammary tissue.[65,66]

The fat in goat's milk can also be modified to produce a value-added product with a more favorable fatty acid profile consisting of higher CLA content. Providing a supplement of 2 and 4% canola oil to lactating goats resulted in an increase of CLA in milk from 10.35 mg/g fat in the basal ration group to 19.4 and 32.0 mg/g fat, respectively.[67]

B. Beef

Factors that affect the yield of CLA in milk should have the same effect on the meat of similar species. The pathway of biohydrogenation in the rumen is well established; however, the effects of polyunsaturated fatty acid (PUFA) levels on the individual enzymes (isomerase and reductase) are not known. It seems that n-3 PUFA having three to six double bonds decreased the efficiency

of biohydrogenation by increasing *trans*-18:1 in the rumen and may facilitate the increased yield of CLA in milk fat. Feeding high levels of α-linolenic acid to sheep is known to increase the rumen content of *trans*-18:1 fatty acids as a result of incomplete biohydrogenation.[68] Feeding whole linseed or fish oil to steers also increased *trans*-18:1 fatty acids in muscle lipids.[69] An increased *trans*-18:1 content in the rumen could be an indication of decreased efficiency of the biohydrogenation process in steps of converting *trans*-18:1 to stearic acid.[70] On the other hand, reduced biohydrogenation efficiency as evidenced by higher *trans*-18:1 could also lead to an accumulation of the biohydrogenation intermediate, CLA, from initial steps in the process. Overall, this would lower the conversion of CLA to *trans*-18:1 and leave more CLA available for incorporation into tissue lipids.

To support this hypothesis, Enser and colleagues[70] showed that both linseed and fish supplements elevated CLA deposition from 5.7 to 8.0 mg/100 g beef muscle. The elevation in CLA was from two- to threefold greater compared with a ration containing more saturated fat (3.2 mg/100 g muscle).[70] The long-chain n-3 PUFA in the fish oil supplement offered to beef cattle was more potent than linseed oil in increasing the CLA deposition in muscle lipid because linseed oil had a greater total amount of n-3 fatty acids (rich in 18:3n-3) than the fish oil (rich in 20:5n-3 and 22:6n-3).

Supplementing cattle with n-3 PUFA could result in a twofold benefit. First, providing cattle rich sources of n-3 PUFA would increase the content of CLA in beef. Thus, with the CLA in milk fat plus that found in beef (30 mg CLA/100 g muscle) from an n-3 fatty acid supplement offered to cattle, the total contribution of dietary CLA would be significantly increased in the food supply. Second, the benefit from CLA is in addition to the elevated level of n-3 PUFA in beef resulting from feeding linseed and fish oil supplements to cattle. The modified beef containing higher levels of both nutraceutical fatty acids is a prime example of a designed food offering health benefits.

Unfortunately, a strong correlation exists between the concentration of CLA and *trans*-18:1 in both milk and beef muscle.[37,70–72] Any increase of *trans*-fatty acids could be of concern since *trans*-18:1 is known to elevate serum levels of low-density lipoprotein (LDL)-cholesterol, a significant risk factor for cardiovascular disease.[73] The small contribution of *trans*-18:1 in beef would be of lesser significance, however, because margarine and food products containing hydrogenated vegetable oils provide far more trans-18:1 to the food supply.

C. Other Animal Derived Designed Foods

Food products derived from nonruminant species such as pork, poultry, and fish can also be enriched with CLA by dietary supplementation. Pigs have been given diets supplemented with CLA to study the effect of CLA on fat-to-lean repartitioning and feed conversion,[74,75] energy balance,[76] and CLA content and isomeric distribution in selected pig tissues.[77] Kramer and colleagues[77] reported that pigs (61.5 to 106 kg live weight) fed a commercial CLA mixture at 2% of the basal diet had CLA isomers enriched in different lipid classes (triacylglycerols, phospholipids, free fatty acids, and cholesterol esters) at levels ranging from 1 to 6% of the total fatty acids in liver, heart, and adipose tissues compared with pigs fed the same basal diet with 2% added sunflower oil, which had only trace amounts (<0.1%) of the 9,11–18:2 *c,t/t,t* isomers in their tissues. The pig adipose tissue contained 4.7% CLA in the inner back fat and 2.9% in the omental fat with an isomeric distribution similar to the CLA source in the diet.

Other investigators have studied the enrichment of CLA in egg yolk and measured the effect of CLA supplementation on egg yolk fatty acid composition and egg quality. In a recent experiment, Watkins and colleagues[45] administered Single Comb White Leghorn hens human-grade soft gel capsules containing 1 g of either CLA (55 to 56% CLA) or safflower oil. The 40 hens were divided into four groups: group 1 served as the control (not supplemented with oil), group 2 received one capsule every other day, group 3 received one capsule every fourth day, and group 4 was given one safflower oil capsule every other day as a sham control. The CLA supplement did not affect hen egg production. However, feed consumption was less in hens supplemented with CLA. Egg yolks from hens treated with CLA were enriched with CLA at amounts that ranged from 0.7 to 1.2% of

total fatty acids containing various isomers (*c,t/t,c* 9,11, *t*10,*c*12, *c*9,*c*11, and *c*10,*c*12). They also observed in this study that the CLA content in the yolk decreased during long-term storage of up to 6 months at refrigerated temperatures (4 to 6°C). Chamruspollert and Sell[43] reported that giving diets supplemented with 5% CLA to laying hens resulted in egg yolk fatty acids that contained 11% CLA isomers. These data indicate that CLA-enriched eggs could be a dietary source of CLA for the human, assuming that egg quality characteristics are acceptable. However, CLA at this level of supplementation increased the firmness of hard-cooked egg yolk. The texture of the yolk from hard-cooked CLA eggs was rubbery and elastic, and the yolks were more difficult to break.[42] Research in our laboratory also showed that egg yolk from hens given a higher level of CLA also led to a rubbery consistency in stored and hard-cooked eggs. Additional research is needed to determine an optimal dietary inclusion of a CLA supplement for commercializing egg products rich in CLA.

Fish are important sources of food protein and beneficial fatty acids that serve as an important link between n-3 PUFA intake and reduced cancer risk and incidence of atherosclerosis.[78] To extend the benefits from fish to another dimension and further improve the quality of fish products, Twibell and colleagues[79] tested the effect of dietary CLA on fish growth, tissue fatty acid composition, and body fat deposition by providing diets containing CLA (0.5, 0.75, and 1.0% of total diet) to juvenile hybrid striped bass. After 8 weeks of dietary treatment, fish given CLA supplements (0.5 and 0.75%) exhibited reduced feed intake without an apparent change in growth rate. However, fish in the 1.0% CLA diet gained less weight compared with fish in the control diet. CLA isomers were detected in liver and muscle of fish fed CLA supplement (see Table 27.2). Total liver CLA concentrations in fish fed 0.5, 0.75, and 1.0% CLA were 2.8, 2.5, and 5.8% of fatty acids, respectively. More CLA was enriched into fish muscle which contained 3.4, 4.4, and 8.1% CLA of total fatty acids, respectively. Fish intraperitoneal fat ratio (intraperitoneal fat × 100/body weight) was significantly decreased in fish fed 1.0% CLA indicating a fat-to-lean repartitioning effect similar to the CLA effect in pigs mentioned above.

D. Vegetable Oils

Creating designed vegetable oils could also be a future dietary source of CLA isomers. The nonselective and selective hydrogenation processes are routinely performed by the edible oils and fats industry with commercially available nonselective and selective catalysts, respectively. With selective hydrogenation, CLA could be enriched in vegetable oil. After 60 min of selective hydrogenation, the fatty acid composition of the partially hydrogenated soybean oil was almost identical to that of the original oil with little change in the iodine value.[80] Therefore, it is possible to produce a high CLA content in soybean oil without altering the concentration of major fatty acids by using a limited (30 to 60 min) selective hydrogenation.

The formation of CLA during hydrogenation is to a great extent dependent on the types and duration of hydrogenation processes. Selective hydrogenation (the conversion of a diene to a monoene fatty acid occurs more selectively compared to the conversion of a monoene to a saturated fatty acid) was much more favorable for the formation of CLA. With selective hydrogenation, the CLA content could reach as high as 98 mg/g oil. The CLA content resulting from these tests using selectively hydrogenated soybean oil was among the highest ever reported in foods.[80] Even though CLA could be produced by this method, the isomeric distribution was totally different from the naturally occurring CLA found in animal-derived foods. Hydrogenated soybean oil in this study contained high levels of *trans,trans* CLA isomers ranging from 32.8 to 60.2% with an increased amount associated with longer hydrogenation times.[80] The CLA isomers *c*9,*t*11 and *t*10,*c*12 both have been proposed to possess beneficial biological effects and accounted for only a combined level of 24 to 57% with equal amounts of *c*9,*t*11 and *t*10,*c*12 isomers in the oil. Also, the percentage of these two isomers decreased with increased hydrogenation time. The value of producing vegetable oils rich in CLA needs to be determined after more information becomes available on the biochemical and physiological effects of individual CLA isomers.

VII. BIOLOGICAL ACTIONS AND POTENTIAL HEALTH BENEFITS OF CLA

CLA is the only known antioxidant and anticarcinogen primarily associated with foods originating from animal sources. Ha and co-workers[10] provided one of the earliest observations that CLA from beef was protective against chemically induced cancer. In that study, CLA isolated from extracts of grilled ground beef was found to reduce skin tumors in mice treated with 7,12-dimethylbenz[α]anthracene (DMBA), a known carcinogen.[10] Since then, numerous investigators have reported the potential beneficial health and biological effects of CLA isomers. The research on CLA has relied almost entirely on animal models and various cell culture systems employing isomeric mixtures of CLA. The properties of CLA include anticarcinogenic[10,59,81–89] and antiatherosclerotic.[90,91] Other CLA effects include antioxidative[81,82,90,91] and immunomodulative.[56,92–95] More recently, preliminary data suggest that CLA may have a role in controlling obesity,[96–98] reducing the risk of diabetes,[99] and modulating bone metabolism.[29,100] In addition, some studies indicate that the biological effects of CLA are influenced by dietary sources of long-chain n-3 fatty acids.[29,95,100]

Extensive work has been done in determining the anticarcinogenic properties of CLA. Isomers of CLA have been shown to reduce chemically induced tumorigenesis in rat mammary gland and colon.[59,82–85,87,89,101] Moreover, CLA modulated chemically induced carcinogenesis in mouse skin[10,102] and forestomach.[81] Sources of CLA also inhibited the growth of human tumor cell lines in culture[88,103,104] and in SCID (severe combined immunodeficient) mice.[86,105]

A. Cancer

In dietary preparations, CLA provided a potent anticarcinogenic effect in a rat mammary cancer model. Ip and colleagues[82] found that administering weanling rats CLA at 0.5 to 1.5% of the diet for 2 weeks before DMBA induction (oral intubation of 10 mg in 1 ml corn oil) and continuing for 36 weeks resulted in a significant reduction in tumor incidence. The effect of CLA was maximized at a dietary level of 1% in this study. In another experiment, much lower doses of dietary CLA (0.05 to 0.5%) were given to rats.[59] The dietary treatments induced a dose-dependent inhibition in mammary tumor yield with a lower dose of 5 mg of DMBA. In the 5-week short-term feeding experiment of 1% dietary CLA,[59] rats were given CLA for 5 weeks and the carcinogens were introduced 1 week before the end of CLA treatment. Total tumor yield was reduced by 39 and 34% in the DMBA and methylnitrosourea (MNU) induction groups, respectively, at the end of the experiment. The short-term feeding period corresponded to the maturation of the rat mammary gland to adult-stage morphology and the supplement of CLA was stopped shortly after the induction with the carcinogens. Therefore, these results suggest that CLA may have a direct modulating effect on susceptibility of the target organ to neoplastic transformation.

In a subsequent dietary study using the rat DMBA model, the inhibitory effect of CLA on induced mammary cancer was found to be independent of fat type (only n-6 and not n-3 fatty acids were investigated) provided in the diet.[83] When providing rats with a CLA supplement, the fatty acid isomers were incorporated into the mammary gland lipids, and the amount in the neutral lipids greatly exceeded that in phospholipids.[84,106] When CLA was removed from the diet after 4 and 8 weeks of feeding, neutral lipid- and phospholipid-CLA returned to basal values in about 4 and 8 weeks, respectively. The author observed that the rate of CLA disappearance in neutral lipids subsequent to CLA withdrawal paralleled closely the occurrence of new tumors in the rat target tissue. It appears that the tissue accumulation of CLA is important to its antitumor effect and that the concentration of CLA in neutral lipid may be a more sensitive biomarker of tumor protection than the amount in phospholipid for this model. The effect of CLA was maximized at a dietary level of 1% as shown in several studies, and increasing the dietary level of CLA did not afford additional protection.[82,106]

The antitumor effect of naturally produced CLA was tested over the chemically prepared CLA mixture from LA which was used in previous studies.[107] During the study, rats were given a diet

with a high-CLA-content butter fat (equivalent to 0.8% CLA in the diet) and a low-level CLA diet that served as a control group (equivalent to 0.1% CLA in the diet) for 1 month before tumor induction using MNU. After tumor induction, rats were changed to a regular diet without CLA for 24 weeks before the termination of the experiment. CLA in butter inhibited the tumor yield by 53% in this study. CLA was also shown to reduce the population of mammary terminal end bud (TEB) cells, the cells that are the primary targets for attack by carcinogens, and the proliferation of TEB cells. Similar effects were reported by Banni and colleagues[89] that providing rats with 0.5 and 1% CLA in the diet produced a graded and parallel reduction in TEB density and mammary tumor yield, and no further decrease was observed in either parameter in rats given 1.5 and 2% CLA. These studies support the hypothesis that exposure to CLA during the time of mammary gland maturation may modify the developmental potential of the target cells that are normally susceptible to carcinogen-induced transformation. In contrast to the previous experiments using a chemically prepared CLA mixture, the latter experiments, for the first time, showed that the natural form of CLA found in dairy foods is active in reducing the incidence of mammary tumors in rats.

The anticarcinogenic properties of CLA isomers on several human cancer cell lines were tested in various cell culture models. Cunningham and colleagues[88] treated human normal mammary cells and MCF-7 breast cancer cells with LA, CLA, and eicosanoid synthesis inhibitors in cell culture. Fatty acids were complexed with bovine serum albumin (BSA) prior to introducing the treatments into the culture media at concentrations of 0 to 3.57×10^5 *M*. The results showed that CLA inhibited thymidine incorporation in normal and MCF-7 cancer cells. The treatment consisting of CLA plus nordihydroguaiaretic acid (NDGA), a lipoxygenase inhibitor, suppressed the growth of MCF-7 cancer cells synergistically. The authors suggested that the growth suppression was augmented by CLA through inhibition of leukotriene synthesis.

The effects of CLA on the growth of three different lung adenocarcinoma cell lines (A-427, SK-LU-1, A549) and one human glioblastoma cell line (A-172) were examined by Schonberg and Krokan.[103] There was a dose-dependent reduction in proliferation of the lung adenocarcinoma cell lines with A-427 being the most sensitive one, but CLA had virtually no effect on the A-172 cell line. In addition, LA had no inhibitory effect on either of these cell lines. A significant increase in lipid peroxidation (measured as formation of malondialdehyde, MDA) was observed after exposure of the lung adenocarcinoma cell lines to 40 μ*M* CLA. The level of MDA was approximately twofold higher than after exposure to 40 μ*M* LA. Treatment with vitamin E (30 μ*M*) totally abolished the formation of MDA; however, the cell growth rates were only partially restored. These data might suggest that cytotoxic lipid peroxidation products are only in part responsible for the growth-inhibitory effects of CLA. Further study is needed to elucidate a possible mechanism of CLA action in lipid peroxidation.

The anticarcinogenic effect of CLA on a human prostatic cancer cell line was investigated in animal models. Cesano and colleagues[86] tested the effect of both CLA and LA against the human prostatic cancer cell line DU-145 in a mouse model. Mice were given a control diet and either LA or CLA at 1% of diet for 2 weeks before the subcutaneous inoculation of cancer cells. Mice were then maintained on the same dietary treatments for 10 more weeks before the termination of the study. Mice given the CLA-supplemented diet displayed not only smaller local tumors than those given the control diet, but also a drastic reduction in lung metastases. Mice given the LA diet had increased local tumor loads as compared with the CLA-treated and control groups. From the aforementioned studies, it is evident that CLA and LA have distinct biological effects.

The anticarcinogenic properties of CLA isomers might be limited to certain types of cancers and may not be effective under some experimental conditions (including dietary sources of phytochemicals and nutraceutical fatty acids such as the n-3 PUFA).[108] Wong and colleagues[109] showed that providing mice diets containing from 0.1 to 0.9% CLA for 2 weeks before infusing with WAZ-2T metastatic mammary tumor cells into the right inguinal mammary gland did not affect mammary tumor latency, tumor incidence, or volume. Therefore, dietary CLA might be less effective on the growth of an established and aggressive mammary tumor.

The anticarcinogenic effects of CLA isomers have been exclusively tested in animal models and cell culture systems. There is no direct evidence that these fatty acids protect against carcinogenesis in humans. The most promising evidence to date that dietary sources of CLA are beneficial is from epidemiological studies that link milk consumption to reduced breast cancer. In one recent study, Knekt and colleagues[110] found a significant inverse relationship between milk intake and breast cancer incidence among 4697 initially cancer-free Finish women over a 25-year follow-up period. It was found that the risk of breast cancer was halved in women who consumed more than 620 ml milk/day compared with those consuming less than 370 ml/day and suggested that CLA was the active component. The average amount of CLA in milk is around 10 mg/g milk fat. Based on the average fat content of 35 g/l milk,[111] the daily intake of CLA for these women could be 217 mg for those consuming 620 ml milk per day and 130 mg for those consuming 370 ml milk per day. However, the estimated CLA intake level for these women is still far below the estimated CLA level of 1 g/day that would be significant for cancer prevention as estimated by Ip and colleagues.[59] The results from the epidemiological studies and reduced incidence of breast cancer could suggest that dairy fats contain other factors that reduce cancer risk or other lifestyle and dietary factors associated with these populations, e.g., fish intake (n-3 fatty acids), that would influence cancer incidence. Experiments designed for investigating the role of CLA in human cancer are needed to identify the relationships between these fatty acids and other lifestyle factors contributing to lower cancer risk.

B. Cardiovascular Disease

The potential health benefits of CLA extend beyond cancer to the prevention of congestive cardiovascular diseases since these fatty acid isomers were shown to reduce atherogenesis in animal studies.[90,91] Lee and colleagues[90] assessed the effect of CLA on atherosclerosis in rabbits by supplementing 0.5 g/animal/day for 22 weeks. Compared with control rabbits that were given a similar diet containing 14% fat and 0.1% cholesterol without CLA, total and LDL cholesterol and triacylglycerol levels in blood were markedly lower in those given CLA. At the same time, the ratio of LDL cholesterol to high-density lipoprotein (HDL) cholesterol and ratio of total cholesterol to HDL cholesterol were significantly reduced in the group given CLA. Rabbits given CLA also showed less atherosclerosis in the aorta. In another study by Nicolosi and colleagues,[91] CLA effects on plasma lipoproteins and aortic atherosclerosis were examined. In the study, 50 hamsters were divided into five groups of 10 and given 0 (control), 0.06 (low), 0.11 (medium), and 1.1 (high) percentage of total dietary energy of CLA, or 1.1% of total energy as LA. Animals given the CLA-containing diets collectively had significantly reduced levels of plasma total cholesterol, non-HDL cholesterol (combined very low-density lipoproteins, or VLDL, and LDL) and triacylglycerols with no effect on HDL cholesterol, as compared with the controls. For the CLA treatment, a tocopherol-sparing effect was observed compared with the control group. Morphometric analysis of aortas revealed less early atherosclerosis in the CLA-treated hamsters compared with the control group. Interestingly, the LA treatment showed a similar effect compared with CLA in this study. From these two studies, CLA was shown to be hypocholesterolemic and antiatherogenic. However, a recent study using C57BL/6 mice given atherogenic diets with added CLA at 0.5 and 0.25% of the total diet demonstrated increased development of aortic fatty streaks despite a change in serum lipoprotein profiles that could be considered less atherogenic.[112]

There is currently no exclusive evidence to support the hypothesis that CLA protects against early atherogenesis. It was initially proposed that CLA was able to protect LDL particles from oxidation[81,82,90,91]; however, a recent *in vitro* investigation by van den Berg and colleagues[19] showed no antioxidant properties attributable to CLA. Also, CLA showed its positive effect on atherosclerosis in rabbits but not in mice, which could indicate a species difference for the response to dietary CLA supplementation.

C. Body Fat and Lipid Metabolism

Numerous animal studies have suggested that CLA could reduce fat deposition and increase lean body mass.[74,75,96–98,113,114] When CLA (0.5 to 1% of diet) was given to AKR/J male mice (39-day-old), it produced a rapid, marked decrease in fat accumulation and an increase in protein accumulation without any major effects on food intake.[96] Park and colleagues[113] provided diets with a lower dietary CLA level (5% corn oil + 0.5% CLA) to 6-week-old ICR mice and observed a significantly reduced body fat content by 57% (male) and 60% (female), and increased lean body mass relative to control mice that were given a diet containing 5.5% corn oil. Similar effects of CLA on body fat accumulation were shown in rats. Yamasaki and colleagues[97] studied the effect of CLA on liver and different adipose tissues, in 4-week-old male rats given diets (AIN-93G with 7% safflower oil as a control) containing 1 and 2% dietary CLA (at the expense of the safflower oil) for 3 weeks. They observed reduced levels of triacylglycerols and nonesterified fatty acids in the liver and white adipose tissue without significant changes in lipids of brown adipose tissue.

Repartitioning of fat to lean was also shown in growing pigs. Dugan and colleagues[74] demonstrated that in pigs (male and female) given a cereal-based basal diet containing either 2% CLA or sunflower oil led to reduced subcutaneous fat deposition and increased lean body mass. Pigs provided with CLA also had reduced feed intake (5.2%) and increased feed efficiency (5.9%) compared with pigs given sunflower oil. In another study, finisher pigs were offered six treatments having from 0 to 10 g CLA/kg diet for 8 weeks.[75] Dietary CLA treatments resulted in increased feed efficiency and lean tissue deposition, and decreased fat deposition (decreased by 31% at the highest CLA level) in growing pigs.

The fat-partitioning effect of CLA was also studied using the adipocyte 3T3-L1 cell culture line. Park and colleagues[113] found that when added during fat accretion in the 3T3-L1 adipocyte culture, CLA (1×10^4 *M* complexed with albumin) reduced lipoprotein lipase activity and enhanced lipolysis leading to less fat deposition. Additionally, skeletal muscle from mice fed CLA exhibited elevated carnitine palmitoyltransferase activity, which indicates elevated β-oxidation of fatty acids. In a recent study using the same cell culture system,[98] a specific CLA isomer, *t*10,*c*12-18:2, was found to reduce lipoprotein lipase activity, reduce intracellular triacylglycerol and glycerol levels, and enhance the release of glycerol into the medium. Based on these findings the physiological mechanism of body fat reduction in mice, and possibly in other animals involving CLA is a result of inhibition of fat storage in adipocytes coupled with both elevated β-oxidation in skeletal muscle and an increase in skeletal muscle mass.[98]

Since adipose tissue is an important site for the regulation of glucose metabolism, and CLA has been shown to modulate the maturation and differentiation of adipocytes and affect lipid metabolism, it is reasonable to assume that CLA could affect glucose metabolism in diabetes. Houseknecht and colleagues[99] tested the effect of CLA on normalizing impaired glucose tolerance in a Zucker diabetic fatty (ZDF) rat model. The ZDF rats were given a diet containing 1.5% CLA for 14 days. At the end of the study, the ZDF rats given the control diet were markedly hyperglycemic compared with ZDF rats given CLA. Moreover, the ZDF rats given CLA had a similar blood glucose level compared with normal lean rats. In this limited study, CLA helped to improve hyperinsulinemia in ZDF rats compared with control rats although the blood insulin level was still ten times more than that of normal lean rats. These results showed that CLA potentially restored cellular sensitivity in liver, muscle, and adipose tissues.

D. Bone

Our laboratory recently investigated the effects of CLA isomers on bone modeling in growing male rats.[29,100] In our studies with rats, 1% dietary CLA, combined with two ratios of n-6/n-3 fatty acids, led to differences in CLA enrichment of various organs and tissues, brain exhibited the lowest concentration of isomers but bone tissues (periosteum and marrow) contained the highest amounts.[29]

Both n-3 fatty acids and CLA lowered *ex vivo* prostaglandin E_2 production in bone organ culture. The supplemental CLA isomers also reduced serum IGF-I concentration and modulated IGFBP differentially depending on the ratio of n-6/n-3 fatty acids in the diet. Moreover, CLA increased IGFBP in rats given a high dietary level of n-6 fatty acids but decreased IGFBP in rats given a high level of n-3 fatty acids. In tibia, rats given CLA had markedly reduced mineral apposition rate (MAR) (3.69 vs. 2.79 μm/day) and bone formation rate (BFR) (0.96 vs. 0.65 μm/day) compared to those not given the CLA supplement.[100] Dietary lipid treatments did not affect serum intact osteocalcin or bone mineral content. These results showed that a mixture of CLA isomers at 1% of the diet modulated local factors that regulate bone metabolism and reduced bone formation rates. This response may be due to the total dietary level of CLA or varying effects of individual isomers of CLA on bone biochemistry and physiology.

In a subsequent study with rats, a 0.5% dietary level of CLA was provided with or without beef fat.[115] The dietary CLA treatments resulted in total CLA values ranging from 0.27 to 0.43% in the polar lipid fraction and from 2.02 to 3.37% in the neutral lipids in liver, bone marrow, and bone periosteum. We observed that CLA accumulated at a higher concentration in neutral lipids compared with polar lipids consistent with the findings of Ip and colleagues[83] for rat mammary gland. In rats, the *t*10,*c*12-18:2 isomer was incorporated into the phospholipid fraction of tissue lipid extracts at the same extent as was the *c*9,*t*11 isomer. The ratio of *c*9,*t*11/*t*10,*c*12 roughly reflected the isomeric distribution of these CLA isomers in the diet or supplement given to rats. Rat serum osteocalcin, a serum bone formation marker, was decreased in rats given CLA after 12 weeks of dietary treatment. Serum bone specific alkaline phosphatase activity was also significantly decreased in rats given CLA. The fact that CLA lowered serum bone formation biomarkers, together with our previous finding that CLA lowered *ex vivo* PGE_2 production in bone organ culture and bone formation rate, suggests that some CLA isomers may exert a downregulatory effect on bone metabolism in growing animals. Future research must be conducted to evaluate the effects of individual CLA isomers on osteoblast function and bone formation.

VIII. MECHANISMS OF CLA ACTION

As the research on CLA developed, several modes of action have been proposed. The CLA isomers have been demonstrated to possess antioxidant properties,[81,82,90,91] inhibit carcinogen–DNA adduct formation,[85,116,117] induce apoptosis,[87] modulate tissue fatty acid composition and eicosanoid metabolism,[29,88,89,94,95,100,118–120] and affect the expression and action of cytokines and growth factors.[95] Since most of the studies mentioned above were conducted with mixtures of several CLA isomers (mainly *c*9,*t*11-18:2 and *t*10,*c*12-18:2), these proposed mechanisms of action might be specific for individual CLA isomers. See Table 27.3 for a summary of these reported actions.

A. Antioxidant

Antioxidants originating from both natural and synthetic sources with diverse structures have been known to demonstrate some anticarcinogenic activity.[121] Since CLA has been proposed to possess antioxidative action, this could mean an important mechanism related to the anticarcinogenic effect of CLA. Several studies have been conducted using both *in vivo* and *in vitro* systems to clarify the role of CLA as an antioxidant; however, the results obtained from different test systems were conflicting. Ha and colleagues,[81] for the first time, showed that CLA was a potent *in vitro* antioxidant. At a very low concentration of 0.375 μmol, CLA inhibited peroxide formation by more than 90% in a reaction mixture consisting of 375 μmol LA, 10 ml of 0.2 *M* phosphate buffer (pH 8), 4.5 ml of water, and 10.5 ml ethanol. CLA was shown to be a more potent antioxidant than α-tocopherol and comparable to butylated hydroxytoluene under the test conditions. Interestingly, in this study a higher CLA concentration did not increase the efficacy but had a diminished antioxidative effect when its concentration was 37.5 μmol.

TABLE 27.3
Reported Chemical, Biochemical, Molecular, and Physiological Actions of CLA

Factor	Action	Organ or tissue	Animal or cell type	Comment	Ref.
		Gene Expression			
Adipose P2 (aP2), PPARγ2, C/EBPα)	Inhibited expression	3T3-L1 pre-adipocytes	Cell culture	CLA inhibited differentiation before confluence and during induction	145
c-myc mRNA	Lowered level			A gene commonly associated with regulating cell cycle components involved in cellular proliferation	120
Stearoyl-CoA desaturase	Decreased Activity	Liver	Mice	This reduction is not the effect of c9,t11 isomer	146
		Enzymes			
Cyanide-insensitive palmitoyl coenzyme A (PcoA) oxidase and carntine acetyl transferase (CAT) activities and total cytochrome P450 (CYP) levels	No effect	Liver	Rat	CLA does not act in the rat as a classical peroxisome proliferator and there may be a difference in the effects on rats and mice	139
Superoxide dismutase, catalase, and glutathione peroxidase	Induced	MCF-7 human breast cancer line and SW480 human colon cancer cell line	Cell culture	CLA induced these antioxidant enzymes. However, the induced enzymes failed to protect cells from cytotoxic lipid peroxidation products	123
Glycerol-3-phosphate dehydrogenase (GPDH)	Inhibited	3T3-L1 pre-adipocytes	Cell culture		145
Ornithine decarboxylase	No change	Dorsal skin	Mice		120
Lipoprotein lipase	Inhibited	3T3-L1 adipocytes	Cell culture	Increased lipolysis in adipose tissue	113
		Cytokines and Growth Factors			
PGE_2	Decreased	Bone organ culture	Rat		29
PGE_2	Decreased	Serum	Rat		94, 118
PGE_2	Decreased	Dorsal epidermis	Mice		120
LTC_4		Lung	Rat		94
TXA_2	Decreased	Human platelet suspension			
IGF-I	Decreased	Serum	Rat		100

continued

TABLE 27.3 (CONTINUED)
Reported Chemical, Biochemical, Molecular, and Physiological Actions of CLA

Factor	Action	Organ or tissue	Animal or cell type	Comment	Ref.
		Cytokines and Growth Factors			
IL-1	Lower	Macrophage	Rat		95
IL-6	Lower	Macrophage	Rat		95
TNF	No change	Macrophage	Rat		95
Leptin	Decreased	Plasma	Mice, AKR/J		96
Insulin	Increased	Plasma	Mice, AKR/J	Increased insulin could lead to increased protein synthesis in muscle	96
Insulin	Decreased	Plasma	Rats, male Zucker diabetic fatty (fa/fa)	CLA normalized impaired glucose tolerance	99
		Peroxidation Products			
TBARS	Increased	MCF-7 and SW480 cell line	Cell culture	MCF-7: human breast cancer line SW480: human colon cancer cell line	123
TBARS	Decreased	Mammary gland	Rat	Maximal antioxidative activity did not parallel with maximal tumor inhibition	82
PLPC oxidation initiated by 2,2′-azo-bis-(2,4-dimethylvaleronitrile) and 2,2′-azo-bis(2-amidinopropane) hydrochloride	No inhibition		Lipid membrane of 1-palmitoyl-2-linoleoyl phosphatidylcholine (PLPC)	CLA did not show a clear protective effect against any of the tested oxidative stress inducers	19
Lipid peroxides	No change	Human mammary epithelial cells and MCF-7 human breast cancer cells	Cell culture		88

Tissue Fatty Acids					
20:4n-6	Decreased	Fat tissue	Chicken		92
20:4n-6	No change	Mammary gland, neutral, and phospholipids	Rat		83
20:4n-6	Decreased	Liver, neutral	Mouse		147
20:4n-6	Lowered	Mammary tissue	Rat		89
Carcinogen-DNA Adduct Formation					
IQ-DNA adducts	Inhibited	Colon	Rat	IQ: 2-amino-3-methylimidazo[*4,5-f*]quinoline PhiP: 2-amino-1-methyl-6-phenyl-imidazo[*4,5-b*]pyridine WBC: white blood cell	85
PhiP-DNA adducst	Inhibited	Liver, WBC	Rat		116
PhiP-DNA adducts and IQ-DNA adducts	Inhibited	Mammary gland, colon	Rat		117
Other					
PPARα		COS-1 cells, CV-1 cells, FaO cells	Cell culture	PPAR: peroxisome proliferator activator receptor; CLA isomers were shown to be PPARα ligands and activators	148
PPARα, β	Activated	Liver	Rats	CLA may regulate hepatic lipid metabolism through a PPAR-mediated mechanism	149
Peroxisome-specific enzyme accumulation and ODC activity	Induced	Liver	Mouse		138
Retinol	Increased	Liver	Rat		150
IgA, IgG, IgM	Increased	Spleen	Rat		94
IgE	Decreased	Spleen	Rat		94

In an *in vivo* study, Ip and colleagues[82] showed that CLA was as effective as vitamin E in inhibiting the formation of thiobarbituric acid reactive substances in the mammary gland but not in the liver. Similar to the results of Ha and colleagues,[81] there was no dose–response relationship in the dietary range of 0.25 to 1.5% CLA. All doses tested produced a 30 to 40% inhibition of peroxide formation. In another dietary study, female rats were given diets containing different lipid types (20% corn oil or lard) or amounts of a fat blend (from 10 to 20% by weight) with or without 1% CLA.[83] The results showed that CLA reduced malondialdehyde (a peroxidation product) production in rat mammary gland homogenate with more reduction found in rats fed high-PUFA corn oil diet (35%) than in those given high-saturated-lard diet (25%). However, CLA failed to show any inhibitory effect on 8-hydroxydeoxyguanosine (8-OHdG, a marker of oxidative damage to DNA) level. Although CLA has been shown to be antioxidative in both *in vitro* and *in vivo* studies, the anticancer effect of CLA in these studies cannot be satisfactorily explained based on this finding. First, the maximum effective concentration of CLA in inhibiting peroxide formation did not agree with the most effective concentration in tumor inhibition.[82] Second, neither of the studies[81,82] demonstrated a dose–response relationship between CLA concentration and its antioxidative efficacy, which is usually true for antioxygenic nutrients studied thus far.

van den Berg and colleagues[19] reinvestigated the antioxidative property of CLA using a lipid membrane system consisting 1-palmitoyl-2-linoleoyl phosphatidylcholine. The results of this study indicated that CLA did not show any protective effect under the test conditions and was more susceptible to oxidative damage than LA and comparable to arachidonic acid. In agreement with the findings of van den Berg and colleagues,[19] Banni and colleagues[122] in a recent study also showed that CLA was more prone to oxidation than LA and no significant antioxidant effect of CLA was detected in the models tested. It would appear that CLA and its metabolites seem to behave like other PUFA under conditions of oxidative stress.

In contrast to the antioxidative properties of CLA, results from experiments using human cancer cell lines suggested that CLA, because of its susceptibility to oxidative damage, could behave as a pro-oxidant in cell culture systems. Moreover, CLA may create an oxidatively stressed environment that is cytotoxic to cultured cells.[103,123] In an experiment O'Shea and colleagues[123] used CLA dissolved in ethanol which was added to a human cell culture (MCF-7 breast cancer cell line and SW-480 colon cancer cell line) media at concentrations of 0, 5, 10, 15, 20, and 30 ppm and incubated for 4, 8, and 12 days. The CLA treatment at 20 ppm increased lipid peroxidation and induced the expression and activity of antioxidant enzymes (superoxide dismutase, catalase, and glutathione peroxidase) in both cell lines. At 20 ppm CLA also reduced ^{3}H-leucine incorporation into protein by 83 to 91% and ^{3}H-uridine and ^{3}H-thymidine incorporation into RNA and DNA by 49 to 91% and 86 to 98%, respectively, compared with untreated control cells. Apparently, at least in part, the induced antioxidant enzyme system failed to protect these cells from peroxidative cytotoxicity. The controversial effect of CLA as an antioxidant or pro-oxidant agrees with previous knowledge that the balance between antioxidant and pro-oxidant activity is known to be a complex function dependent on the concentration of and oxygen partial pressure.[124]

B. Biochemical and Physiological Actions

Another potential action of CLA in preventing carcinogenesis is on DNA adduct formation. For example, dietary CLA (1, 0.5, 0.1% diet) inhibited PhIP (a mammary carcinogen)–DNA adduct formation in F344 rat liver and white blood cells in a dose-dependent manner.[116] Similar finding were also reported by Liew and colleagues[85] that when F344 rats were given CLA (0.5% diet equivalent) by gavage the treatment significantly lowered IQ (2-amino-3-methylimidazo[4,5-*f*]quinoline)–DNA adduct formation in the colon. Schut and colleagues[117] used the same rat model to study CLA effects on both PhIP and IQ and found that CLA (0.1 to 1%) inhibited PhIP-DNA adduct formation in mammary gland and the colon. It was concluded that CLA could be a potential chemopreventive agent against PhIP- or IQ-induced tumors in rodents.

The isomers of CLA might also initiate apoptosis to protect mammary gland cells from chemically induced carcinogenesis. Ip and colleagues[87] showed in a primary culture of rat normal mammary epithelial organoids (MEO) that CLA (0 to 128 μ*M*), but not LA, inhibited growth of MEO which was further shown to be mediated by a reduction in DNA synthesis and a stimulation of apoptosis. By inducing apoptosis in mammary gland epithelial cells, CLA could prevent breast cancer by reducing mammary epithelial cell density and inhibiting the outgrowth of initiated MEO.

Isomers of CLA may exert their biochemical and physiological effects by modulating tissue or cellular eicosanoid metabolism.[29,88,89,94,95,100,118–120] In studies to determine the physiological action of CLA, investigators found reduced prostaglandin E_2 (PGE_2) (a cyclooxygenase, or COX, catalyzed product of arachidonic acid) concentration in rat serum and spleen.[94,118] The amount of PGE_2 in cultured keratinocytes[120,125] and in *ex vivo* bone organ culture[29] were lowered with CLA treatment in cells and rats, respectively. Moreover, CLA reduced the level of leukotriene B_4 (LTB_4) from the exudates of cells[94] and thromboxane A_2 (TXA_2) (another cyclooxygenase catalyzed product of arachidonic acid) in platelet suspension.[126] Indirectly, CLA was found to suppress the growth of human MCF-7 breast cancer cells in culture synergistically with nordihydroguaiaretic acid (NDGA, a lipoxygenase inhibitor),[88] indicating an inhibitory effect of CLA on the lipoxygenase pathway.

The mixed isomers of CLA were detected in numerous tissues examined in animals given a dietary supplement of CLA.[29,118] In one of our experiments, four groups of male rats were given a basal semipurified diet (AIN-93G) containing 70 g/kg of added fat for 42 days.[29,100] The fat treatments were formulated to contain two levels of CLA (0 and 1% diet) and either n-6 (soybean oil having a ratio of n-6:n-3 fatty acids of 7.3) or n-3 fatty acids (menhaden oil + safflower oil having a ratio of n-6:n-3 fatty acids of 1.8) following a 2 × 2 factorial design. The fatty acid composition of liver, serum, muscle, heart, brain, spleen, and bone (cortical, marrow, and periosteum) were analyzed by GC. The CLA isomers were found in all rat tissues analyzed although their concentrations varied with brain exhibited the lowest concentration of CLA isomers but bone tissues (periosteum and marrow) containing the highest amounts. Dietary CLA decreased the concentrations of 16:1n-7, 18:1, total monounsaturates and n-6 fatty acids, but increased the concentrations of n-3 fatty acids (22:5n-3 and 22:6n-3), total n-3 and saturates in the tissues analyzed. *Ex vivo* PGE_2 production in bone organ culture was decreased by the n-3 fatty acid and CLA treatments. In a subsequent study with rats, feeding 0.5% CLA resulted in total CLA concentrations ranging from 0.27 to 0.43% in the polar lipid fraction and from 2.02 to 3.37% in the neutral lipids in liver, bone marrow, and bone periosteum. Our finding that the CLA isomers accumulated at a higher concentration in neutral lipids compared with polar lipids is consistent with that of Ip and colleagues[83] for rat mammary gland. Moreover, the *trans*-10,*cis*-12 18:2 isomer was incorporated into the phospholipid fraction of tissue lipid extracts at the same extent as was the *cis*-9,*trans*-11 isomer. The ratio of *cis*-9,*trans*-11/*trans*-10,*cis*-12 in polar lipids roughly reflected the isomeric distribution of these CLA isomers in the dietary supplement given to rats.

When CLA is incorporated into membrane phospholipids (induced by feeding a dietary supplement), it may compete with arachidonic acid, and is likely to inhibit eicosanoid biosynthesis.[100,127,128] The reduction of the proportions of n-6 fatty acids in peritoneal exudate cells and splenic lymphocyte total lipids by CLA seemed to be responsible at least in part for the reduced eicosanoid levels.[94] CLA could also affect the lipoxygenase pathway to reduce product formation. Cunningham and colleagues[88] showed that the addition of CLA and NDGA to MCF-7 cells resulted in synergistic growth suppression suggesting that CLA effects were mediated through lipoxygenase inhibition.

The effect of dietary CLA on eicosanoid metabolism could be twofold. First, CLA isomers could directly compete with substrate concentration and activity or expression of cyclooxygenase or lipoxygenase. Second, CLA could be further desaturated and elongated to its 20-carbon equivalent, and exert its effect by competing with other 20-carbon fatty acids, mainly arachidonic acid and eicosapentaenoic acid (EPA) to reduce the production of their corresponding eicosanoids. Experiments to evaluate the effects of CLA isomers on eicosanoid biosynthesis have not yet been conducted. We hypothesized that CLA depressed arachidonate-derived eicosanoid biosynthesis

since dietary sources consistently reduced *ex vivo* PGE_2 production in rat bone organ culture and liver homogenate.[100] The reduction in PGE_2 by CLA might be explained as a competitive inhibition of n-6 PUFA formation that results in lowered substrate availability for cyclooxygenase. Although there was a trend of reduced arachidonic acid concentration in bone tissues, the dramatic decrease in *ex vivo* PGE_2 production in bone organ culture could not be satisfactorily explained by a lack of substrate.

The biosynthesis of PGE_2 in bone (cells of the osteoblast lineage) is highly regulated by local and systemic factors.[129–131] Fatty acids have been shown to modulate the expression and activity of this key enzyme. For example, Nanji and colleagues[132] showed that saturated fat reduced peroxidation and decreased the levels of COX-2, the inducible form of COX, in rat liver. In a rat dietary study on colon tumorigenesis, a high-fat corn oil diet (rich in n-6 fatty acids) upregulated COX-2 expression, but a high-fat fish oil diet (rich in n-3 fatty acids) inhibited it; however, expression of COX-1, the constitutive enzyme, was not affected.[133] We speculate that CLA may influence PGE_2 production through the COX enzyme system, more likely on COX-2, to exert its physiological effects in bone and other tissues to influence bone metabolism as well as cancer.

Sebedio and colleagues[119] reported that CLA may be further desaturated and elongated to form conjugated 20:4 isomers which might block the access of arachidonic acid to COX. The unusual 20:4 isomers derived from CLA might also affect the activity of the COX enzymes. Further study with CLA is needed to confirm if its isomeric analogues affect COX activity and expression as a primary mechanism of action and potential role in controlling cancer and inflammatory disease.

The isomers of CLA were shown to modulate the expression and activity of cytokines and growth factors. We reported that dietary CLA lowered basal and lipopolysaccharide (LPS) stimulated IL-6 production and basal TNF production by resident peritoneal macrophages in rats.[95] Furthermore, CLA reduced the release of LTB_4,[94] a strong bone resorption factor,[134] from peritoneal exudate cells and splenic cells in response to the dietary CLA levels. Assuming that CLA would have similar effects on these cytokines in bone, together with the fact that CLA reduced the production of PGE_2 in bone tissue, one could hypothesize that at a proper dietary level, the anti-inflammatory effects of CLA would be beneficial for the treatment of inflammatory bone diseases.

In our laboratory, the dietary CLA effects on serum concentrations of IGF-I and IGF-binding proteins (IGFBP) and their subsequent impact on bone modeling were examined in male rats. The level of IGFBP in serum of rats was altered by n-6 and n-3 fatty acids but CLA had variable effects. Interestingly, CLA increased IGFBP level in rats given a high dietary level of n-6 fatty acids but reduced it in those given a high level of n-3 fatty acids. Rats given the n-3 fatty acids had the highest serum level of IGFBP-3. This study also showed that CLA decreased the amount of IGF-I mRNA in liver of rats given n-3 fatty acids. In liver of rats, the expression of IGF-I mRNA appeared to be upregulated by n-3 fatty acids and downregulated by CLA. The lowering effect of CLA on growth factors was associated with reduced mineral apposition rate and bone formation rate in the tibia. These results showed that dietary PUFA type (and level) and CLA modulate growth factors that regulate bone metabolism.

Different CLA isomers may also have their own unique mechanisms of action. Although limited information is available on the biological activity of individual CLA isomers, the data are suggestive that individual isomers exert different biological effects. Currently, the *c*9,*t*11 isomer has been shown to be effective in reducing carcinogenesis and the *t*10,*c*12 isomer is more potent in inducing body compositional change. de Deckere and colleagues[135] treated hamsters with CLA preparations containing relatively pure *c*9,*t*11, *t*10,*c*12, or a mixture of both isomers and showed that the *t*10,*c*12 isomer was the most active form of CLA in inducing biological effects such as increasing liver weight, decreasing fat deposition, and lowering LDL cholesterol. The authors concluded that t10,c12 appeared to be the physiologically active CLA isomer and the natural *c*9,*t*11 had little or no effect on lipid metabolism in hamsters.

Studies performed on mouse and cell culture also indicate differences between the two major CLA isomers in their clearance rate from the body. In skeletal muscle of mice treated with dietary

CLA supplements, the *t*10,*c*12 isomer was cleared significantly faster than the *c*9,*t*12 isomer.[114] Yotsumoto and colleagues[136] recently showed that *t*10,*c*12, but not the *c*9,*c*11 isomer or LA, inhibited cellular triacylglycerol synthesis and reduced apolipoprotein B secretion in HepG2 cell cultures. In the same study, the *c*9,*c*11 isomer inhibited cholesteryl ester synthesis but at a lesser extent compared with the *t*10,*c*12 isomer.[136] Information on the effects of individual CLA isomers is inadequate at present time because of the limited supply of pure CLA isomers. With the advance in techniques on CLA preparation, more pure CLA isomers will be available for future research.

IX. POTENTIAL ADVERSE EFFECTS OF CLA

Since CLA has become a widely advertised nutritional supplement for human use,[137] it is important to study both its positive and its potential negative effects on health using cell culture or animal models. Our recent dietary studies using an isomeric mixture of CLA revealed a negative effect on rat bone metabolism.[100,115] Rats given 1% CLA in the diet demonstrated decreased mineral apposition rate and bone formation rate in the tibia compared with rats not given CLA. In another study with rats a lower level of CLA (0.5% diet) reduced serum osteocalcin and bone-specific alkaline phosphatase activity, both biomarkers of bone formation, after 12 weeks of dietary treatment (unpublished results). The negative effect of CLA was likely due to a high dietary level of isomeric mixtures that do not reflect the usual food sources of CLA.

In a recent study by Belury and colleagues,[138] CLA displayed the typical peroxisome proliferation response in rodent liver. Peroxisome proliferators may enhance tumorigenesis in liver, testes, and pancreas by acting as promoters, resulting in enhanced cell proliferation, altered cell differentiation, and inhibition of apoptosis in initiated cells.[138] This response might suggest that the chemoprotective effect of CLA in extrahepatic tissues[82,83,85,102] may be at the expense of enhanced hepatocarcinogenesis. Jones and colleagues[139] also reported that CLA lowered blood LDL cholesterol but increased VLDL cholesterol and resulted in liver hypertrophy in mice.

The results of Belury and colleagues[138] indicate that the peroxisome proliferation response to CLA may be greater in mice than in rats. These data suggest a species difference in the response to CLA and such information might be relevant to a proper risk assessment for human consumption of isomeric mixtures of CLA. Thus far, the information on CLA action in humans is still very limited and more research is necessary to clarify safety issues related not only to isomeric supplements of CLA, but also to individual isomers that could behave differently from each other in various biological systems and physiological conditions.

Many CLA isomers present in commercial CLA supplements for human use do not exist in natural food products. Furthermore, even for the naturally occurring CLA isomers, human consumption without dietary supplementation is normally at a very low level. The possible negative effects of CLA at trace levels could not be easily detected. Therefore, before promoting the human use of dietary CLA supplements, thorough examination of the effects of individual CLA isomers is necessary to protect consumers from potential detrimental effects. Together with the fact that CLA lowered serum bone formation biomarkers and bone formation rate, we believe that specific research must be implemented to evaluate the potential safety questions on these promising chemopreventive agents.

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28 The Role of Nuts in Cardiovascular Disease Prevention

Joan Sabaté, Timothy Radak, and Jack Brown, Jr.

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I. INTRODUCTION

Since preagricultural times, nuts have been eaten as part of the human diet,[1] providing a complex plant food rich in macro- and micronutrients and fiber as well as other bioactive constituents, most of them as yet undiscovered. While it could be postulated that nuts once made up a sizable portion of the diet, today that is surely not the case. According to data from the 1980s nut consumption appears to be on the decline.[2] There are exceptions in a few distinct health-conscious groups or regions,[3,4] such as vegetarians who regularly consume nuts in part as a source for protein, religious groups such as the Seventh-Day Adventists who are also mostly vegetarian, and in countries that follow a Mediterranean diet, where consumption is twice that found in the American diet.[2]

This record of abstemious consumption of nuts relative to other foods is most likely derived from the commonly held belief arising from public health messages (1) to reduce fat intake, and (2) that high-fat containing foods should be used sparingly and in moderation. This recommendation has been practically translated into an obsession of the public to choose low-fat foods and to decrease the intake of high-fat products. This was due to the implication of fat as a risk factor for chronic diseases, such as cardiovascular disease and some forms of cancer, both leading causes of

0-8493-8734-5/01/$0.00+$.50
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death in the Western world. The risk of obesity, also a serious health concern linked with chronic disease, has spurred the public to focus on limiting fat intake even further, which virtually guaranteed a less than lackluster image for nuts, as they are on average 59% fat by weight. Nuts are thus seen as special foods, considered more of a luxury food with no beneficial health effects.

Americans on the whole are acclimating to a lower-fat diet. Paradoxically, despite these recommendations, the prevalence of obesity in the United States continues to increase. Although total fat intake certainly has a role, research is now nearing agreement that it is not just total intake of fat that is of concern, but rather that the type of fat or fatty acid may be related to the rise in obesity, as well as being a risk factor associated with chronic disease. It is also well established that the fatty acid composition, rather than the total amount of fat consumed, is a greater predictor of cholesterolemic effects. Unlike animal-derived foods, which are a main source of saturated fat and offer little fiber, nuts comprise mainly unsaturated and polyunsaturated fatty acids and are a good source of fiber. This difference has focused new attention on the investigation and effects of nut consumption in relation to lessening coronary disease risk and has further prompted the hypothesis of the way diet can influence plasma lipid values.[5]

Nuts, as shall be seen, have a direct bearing on all of these factors, and may bring new meaning to nuts being looked at as "special." As so much has been recommended on what not to eat, carrying with it negative associations, the potential of nuts as a functional food that offers positive health effects could bring a welcoming message for a society increasingly concerned about health and chronic disease. Many are taking an active role in being accountable for their health and taking dietary steps to lessen disease risk. Indeed, it is becoming evident to the public, in addition to the scientific community, that dietary practices play a significant role in influencing disease outcome and risk. This chapter explores where the attention involving nuts has been focused, what mechanisms may be involved, and where research efforts should be directed in validating the use and consumption of nuts as a functional food and as a possible dietary means for disease prevention.

II. NUTRIENT COMPONENTS AND COMPOSITION OF NUTS

Table 28.1 summarizes the fat content of several nuts. By weight, the total fat content of nuts is high, ranging from 45 to 74%, but this fat is largely unsaturated. Most nuts are high in monounsaturated

TABLE 28.1
Fat Composition of Several Nuts

		Percentage of Total Fat				
	Total Fat (% of weight)	SFA	MUFA	PUFA	PUFA/SFA Ratio	UFA/SFA Ratio
Almonds	52	10	68	22	2.2	9.0
Brazils	66	26	36	38	1.5	2.8
Cashews	46	20	62	18	0.9	3.9
Hazelnuts	63	8	82	10	1.2	11.9
Macadamias	74	16	82	2	0.1	5.4
Peanuts	49	15	51	34	2.3	5.7
Pecans	68	8	66	26	3.2	10.9
Pinyons	61	15	40	45	2.9	5.4
Pistachios	48	13	72	15	1.2	6.6
Walnuts	62	10	24	66	6.5	9.0

Note: SFA denotes saturated fatty acids; MUFA denotes monunsaturated fatty acids; PUFA denotes polyunsaturated fatty acids; UFA denotes unsaturated fatty acids (MUFA plus PUFA).

Source: Based on data from the USDA Handbook No. 8–12.

fat ranging from 24 to 82% of total fat, except for walnuts, which are high in polyunsaturated fatty acids, around 66%. The ratio of unsaturated to saturated fatty acids, up to 11.9, is extremely high compared with other fatty foods. However, nuts are more than just bundles of fat, and presented in Table 28.2 is a summary of the selected nonfat nutrient components of nuts, revealing the high protein, high fiber, vitamin, and mineral content of most nuts. Nuts also contain numerous phytochemicals. Unfortunately, there are limited data on plant chemicals in nuts at the present time. As will be discussed in a later section, some of these components have been hypothesized as potential mechanisms in reducing coronary heart disease (CHD) risk.

III. NUT CONSUMPTION AND CHD: EPIDEMIOLOGICAL EVIDENCE

Traditionally, nutrients rather than individual foods have been studied in relation to CHD. Foods, including nuts, contain both nutrients and non-nutrient chemicals which have unknown biological activity; it is therefore important also to study the effects of natural foods on risk factors of chronic disease. Potentially, these findings may be more applicable to the public for incorporation into dietary practice.

The few studies that have looked at the consumption of whole nuts in relation to CHD have reported a consistent and substantial protective effect. Three of the largest nutritional epidemiological prospective studies spanning diverse populations, in age, race, gender, health status, and occupation, have produced consistent findings in relationship to nut consumption and coronary risk.

A. The Adventist Health Study

The Adventist Health Study (AHS)[6] was the first to generate interest in the use of whole food nuts as they relate to CHD. The first 6 years of follow-up in this prospective study provided the opportunity to follow 31,000 non-Hispanic Caucasian Seventh-Day Adventists living in California to investigate the relationship between certain foods and chronic diseases. The cohort is unique in its homogeneity in that the Adventist population is well educated and highly interested in diet and health matters. There is virtually no prevalence of cigarette smoking and minimal use of alcohol, thus limiting the effects of these as potential confounders. Approximately 50% of Adventists adhere to a lacto-ovovegetarian diet, thus making determinations for meat and nonmeat intake. This makes the subjects highly suitable for studying dietary relationships in light of other environmental effectors.

A food frequency questionnaire made inquiry of 65 food items, one of which was the use of nuts. Approximately 10,000 men and 16,700 women ranging in age from 25 to 101 years had dietary and lifestyle habits recorded. Annual questionnaires and medical report findings followed up CHD data for 6 years. Independent relationships between intake of several foods at baseline and the end points of nonfatal myocardial infarction or death from CHD were considered. Data were stratified according to age, sex, smoking, exercise, relative weight, and hypertension to control for confounders. Analysis was done in the whole population of the consumption of eggs, milk, and meat, along with the standard CHD risk factors.

Frequency of nut consumption was inversely related to the risk of having an acute myocardial infarction or dying of CHD. Nuts had one of the strongest inverse relationships out of all the foods evaluated. As seen in Figure 28.1, those who ate nuts one to four times per week had a 22% reduced risk of acute myocardial infarction (P for trend <0.005), whereas those eating nuts five or more times per week showed a 51% reduction in risk (P for trend <0.001).[7] Similar results were found with death from ischemic heart disease.

After multivariate analysis, the strong inverse relationship between nuts and CHD risk remained statistically significant, furthering the unlikelihood that these results were due to chance. The protective effect of nuts was seen in both vegetarians and nonvegetarians (Figure 28.2), indicating the beneficial effects seen from nut consumption were independent of meat intake. In this population, nut consumption seems to confer protection against CHD, even in the oldest group (those over age 84).[8]

TABLE 28.2
Nonfat Components of Nuts with Hypothesized Antiatherogenic Properties

	γ-Tocopherol (mg)	Dietary Fiber (g)	Protein (g)	Lysine (g)	Arginine (g)	Lys/Arg	Mg (mg)	Cu (mg)	Folate (μg)
Almonds	24.0	11.2	20	0.7	2.5	0.3	296	0.9	58.7
Brazils	7.6	5.4	14	0.5	2.4	0.2	225	1.8	4.0
Cashews	0.6	6.0	15	0.8	1.7	0.5	260	2.2	69.2
Hazelnuts	23.9	6.4	13	0.4	2.2	0.2	285	1.5	71.8
Macadamias	--	5.2	8	0.3	0.9	0.4	116	0.3	15.7
Peanuts	9.1	8.8	26	1.0	3.5	0.3	180	1.0	239.8
Pecans	3.1[a]	6.5	8	0.3	1.1	0.3	128	1.2	39.2
Pinyons	—	—	12	0.4	2.3	0.2	234	1.0	—
Pistachios	5.2	10.8	21	1.3	2.2	0.6	158	1.2	58.0
Walnuts	2.6[a]	4.8	14	0.4	2.1	0.2	169	1.0	66.0

Note: Values per 100 g of edible food.

[a] Pecans contain 19.1 mg of γ-tocopherol; walnuts contain 17.2 mg.

Source: Based on date from USDA Handbook No. 8-12.

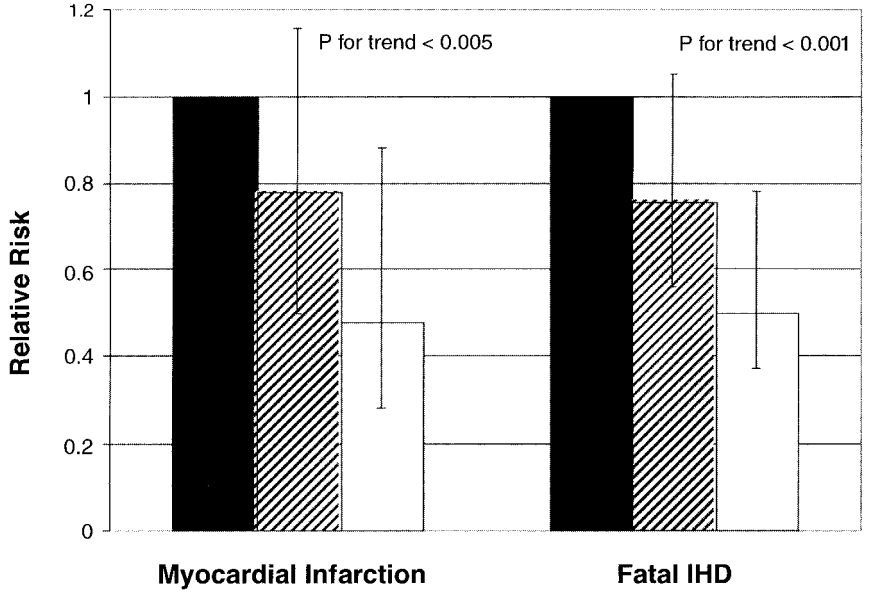

FIGURE 28.1 Adjusted relative risk of two ischemic heart disease (IHD) end points according to frequency of nut consumption (black, <1 time/wk; lined, 1–4 times/wk; white, ≥5 times/wk): the Adventist Health Study. Data adjusted simultaneously for age, sex, smoking, exercise, high blood pressure, consumption of meat, and bread type. Vertical lines indicate 95% CIs.

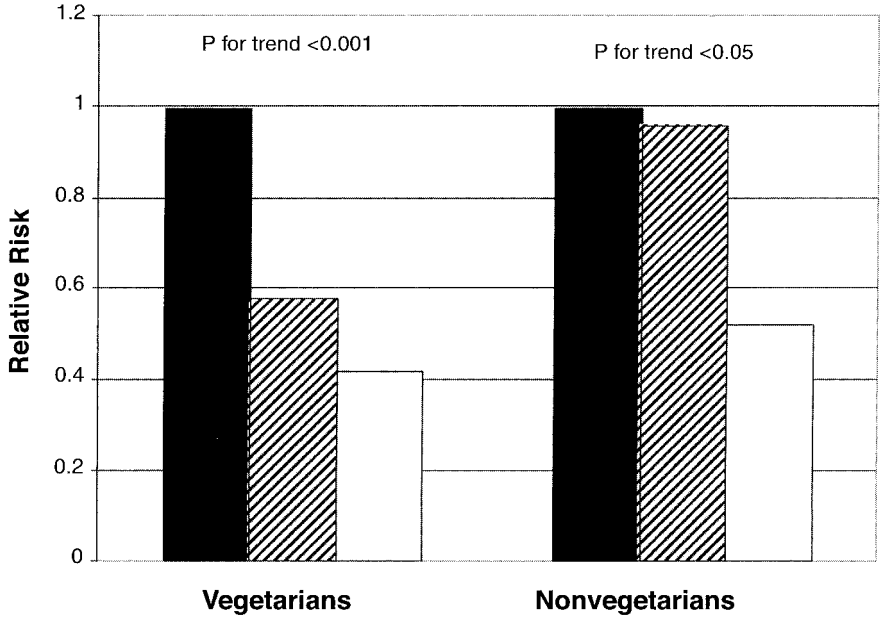

FIGURE 28.2 Age- and sex-adjusted relative risk of definite IHD events at three levels of nut consumption (black, <1 time/wk; lined, 1–4 times/wk; white, ≥5 times/wk), according to vegetarian status: the Adventist Health Study.

Subsequent publications from the AHS assessed the lifetime risk of developing CHD and computed age at first coronary event. Lifetime risk of CHD development was reduced 12% in the group that ate nuts five or more times per week, and men who did develop the disease did so 5.6 years later than men who ate nuts infrequently.[9] These results suggest that high nut consumption not only decreases the risk of developing CHD, but also postpones the development several years in those who acquire the disease.

B. The Iowa Women's Health Study

The Iowa Women's Health Study[10] looked at 34,000 postmenopausal women aged 55 to 69 years. This 7-year prospective study assessed by questionnaire the intake frequency of 127 foods, including nuts, as well as lifestyle questions relevant to CHD. Women in Iowa had a greater number of recognized CHD risk factors, such as hypertension, smoking, and higher body mass indexes, than in the California Adventists.

Nut consumption in this population was considerably lower than in the Adventist cohort: 41% never ate nuts, 35% ate them one to three times per month, 15% ate them once per week, and 9% ate nuts two or more times per week.[4] These levels are more representative of general vs. population intake.

Despite the difference in nut consumption as compared with the women in the AHS, the results were very similar. In multivariate analysis, which adjusted for traditional risk factors and energy intake, an inverse association between nut and seed consumption and risk of death from CHD was seen. The highest nut consumption category conferred a 40% reduced risk of CHD compared with women who never ate nuts (P for trend = 0.016, RR [relative risk] 0.60, and 95% CI [confidence interval] 0.36 to 1.01). Many nuts are a source of α-tocopherol. Additional adjustment for vitamin E intake lessened the association to 28%, inferring its contribution to the reduction. Interestingly, consuming nuts one to three times per month offered no appreciable benefit for CHD reduction.[7]

C. The Nurses' Health Study

The Nurses' Health Study,[11] another prospective study, also investigated the same relationship as the above two studies and found corresponding evidence. The largest of the three studies, it looked at 86,000 registered female nurses aged 34 to 59 years, spanning several states. The results shown here for middle-aged women confirm the previously published studies, which had included older women and men, illustrating that the protective effect of nut consumption extends across gender and age groups.

Beginning in 1976, detailed information on medical history and lifestyle was established, and follow-up questionnaires were distributed every 2 years thereafter for a total of 14 years. A dietary questionnaire of 61 items was added after 1980 to assess intake of nuts as related to other foods and nutrients. Four categories of nut consumption were considered (with 1 time being equivalent to 1 oz of nuts): almost never, 1 to 3 times per month to once per week, 2 to 4 times per week, and ≥5 times per week. Data were obtained for fatal and nonfatal myocardial infarction for the 14 years of follow-up.

As mentioned, results confirmed findings in previous studies. After adjusting for traditional risk factors, there was a 39% reduction (P for trend = 0.007, RR 0.61, and 95% CI 0.35 to 1.05) for fatal CHD, and 32% for nonfatal myocardial infarction (P for trend = 0.04, RR 0.68, and 95% CI 0.47 to 1.00) for those consuming nuts five or more times per week compared to those eating nuts less frequently, less than once per week. After adjusting for age, smoking, and other traditional CHD risk factors, women in the ≥5 times per week category had a significantly lower risk of total CHD (P for trend = 0.0009, RR 0.65, and 95% CI 0.47 to 0.89) compared with those in the <1 time per week category.

Further adjustment for dietary intakes of fats, fiber, vegetables, and fruits did not diminish the outcomes. The results did not change appreciably even after 32 subgroups were stratified for

potential confounders; for example, by levels of smoking, use of alcohol, multivitamin and vitamin E supplements, body mass index, exercise, and intake of vegetables or fruits. All subgroups showed an inverse association. In 25 and 29 of the 32 subgroups, the *P* for trend was <0.05 or <0.01, respectively.

As with any epidemiological study, there is always the possibility of effects resulting from confounding. For example, in this study it was found that women who consumed more nuts also had a healthier lifestyle and diet than those who consumed nuts rarely or not at all. When adjusting for these differences, a predictible attenuation resulted. However, even after such adjustments, a clear and statistically significant result remained. Given this, the authors argue that although residual confounding cannot be ruled out, it is unlikely that it can fully explain the observed strong inverse association.

Figure 28.3 shows the graph for the relative risks based on frequency of nut consumption for all of the above three epidemiological studies. It illustrates that although these cohorts had different distributions in nut consumption frequency and cutoff points, it is possible to observe together the effects of nuts over a wider range of intake. Trends can be surmised and a graded relation easily seen. Again, the results from these three published studies were remarkably consistent.

An additional cohort study in males with findings in abstract form found results similar to the above three studies. The Physicians Health Study[12] looked at 22,000 male physicians over a 12-year follow-up examining food intake. A significant decrease in the risk of sudden and cardiac death as nut consumption increased was observed. This inverse relationship persisted after adjustment for age, exercise, hypertension, smoking, cholesterol, diabetes, alcohol, and other dietary habits.

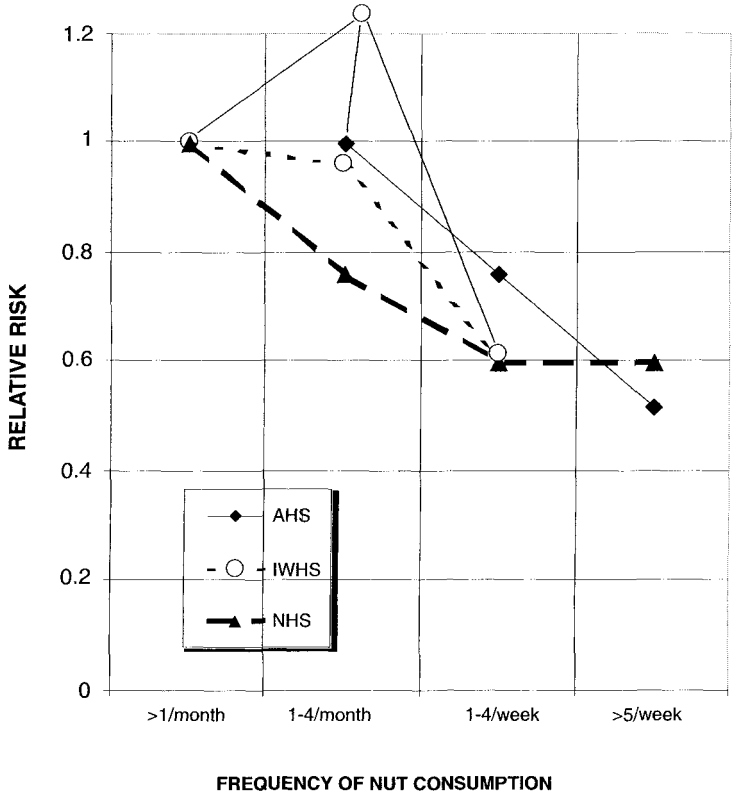

FIGURE 28.3 Relative risk of IHD according to frequency of nut consumption in three large cohort studies: Adventist Health Study (AHS), Iowa Women's Health Study (IWHS), and the Nurses' Health Study (NHS).

D. All Cause Mortality and Nut Consumption

What are the possible effects of nut consumption on all-cause mortality? Researchers of the AHS looked at all-cause mortality across differing gender, racial, and age groups, and it was shown to be inversely related with the frequency of nut consumption on these Adventist subpopulations.[8,13,14] Although the reasons for this association are not entirely understood, it is presumed that it is reflective of the decrease in CHD mortality. Protection of CHD by nut consumption is not offset by an increased mortality from cancer,[14] or competing causes.[9] As Table 28.3 shows, lower mortality was seen in Caucasians, African Americans, and elderly groups.[7] There was an 18 to 44% reduction, depending on the group and level of adjustment in those that ate nuts five or more times per week, compared with those that ate nuts fewer than one time per week. These effects again seem to be independent of other risk factors and suggest that nut consumption not only decreases risk from CHD, but may offer an extension in longevity as well.

E. Secondary CHD Prevention

One other epidemiological study was unique in that it examined the protective effect of nuts on a population who had already experienced a myocardial infarction. Brown et al.[15] looked at postmyocardial infarction in men and women aged 21 to 75 years who were hypercholesterolemic for an average of 4.2 years of follow-up. A food frequency questionnaire was administered. Those who had frequent consumption of nuts (≥2 servings per week) had a 25% lower risk of total CHD compared with those who rarely ate nuts (<1 serving per month). This 25% reduction in risk was observed after adjustment for age, smoking, and other traditional CHD risk factors. When looking for possible nutrients that could be involved in explaining the reduction seen, unsaturated fatty acids, minerals, and antioxidant vitamins were adjusted for and did not appreciably alter the findings.

F. Summary of Epidemiological Findings

Combined, these five epidemiological studies offer evidence of a significant protective benefit to fatal and nonfatal CHD events, possibly recurrent CHD events, and even increased longevity from the inclusion of whole food nut consumption in the diet. That the results showed a strong, consistent decrease in CHD risk across subpopulations of males and females, differing ages, geographic locations, and occupations is compelling and distinguishes itself from research on other food items that have shown less uniform and consistent findings. Remarkable as the findings may be, it precludes definitive proof, as the potential for residual confounding cannot be completely excluded in nutritional epidemiological research, unlike randomized controlled trials, which can approach causality.

IV. NUT CONSUMPTION AND BLOOD LIPIDS: A CROSS-SECTIONAL SURVEY

In a recent cross-sectional survey done in France, the consumption of walnuts and walnut oil was related to blood lipids in a population that typically consumes walnuts as part of their standard diet.[16] Approximately 800 men and women between the ages of 18 and 65 years reported nut consumption using food frequency and dietary recall methods, and had blood samples drawn. After controlling for gender, body mass index, dietary animal fat, and alcohol, a modest but significant increase in serum high-density lipoprotein (HDL) cholesterol and Apo protein A1 was observed among frequent walnut consumers compared with nonconsumers. No changes were seen for low-density lipoprotein (LDL) cholesterol or triacylglyceride serum levels for either walnut oil and walnut or walnut consumers as compared with nonconsumers.

These positive findings, although suggestive, could be partially explained by the resulting increase in total dietary fat among frequent walnut and walnut oil consumers. Furthermore, the type of study design (crossover) prevents one from drawing causal relationships. Nutritional studies

TABLE 28.3
Frequency of Nut Consumption and All-Cause Mortality in Several California Seventh-Day Adventist Subpopulations

Ref.	Population	Follow-Up Period	Adjustments	Hazard ratio (95% CI) for nut consumption		
				<1 time/wk	1–4 times/wk	≥5 times/wk
14	Whites (n = 31208)	1976–1982	Age, sex, and vegetarian status	1.0	0.72	0.67[a]
13	Blacks (n = 1668)	1976–1985	Age, sex, smoking, and exercise	1.0	0.60 (0.4, 0.9)	0.56 (0.3, 1.0)
8	Oldest to old, >84 years of age (17,446 person-years)	1976–1988	Age, sex	1.0	0.82 (0.72, 0.95)	0.75 (0.65, 0.86)
			Age, sex, diabetes, smoking, exercise, and meat, fish, fruit, bread, doughnut, and sweets consumption	1.0	0.88 (0.79, 1.01)	0.82 (0.70, 0.96)

[a] Significant trend $P < 0.001$.

specifically designed to examine the effect of specific nut consumption on serum lipid levels are presented in the following section.

V. HUMAN NUTRITIONAL STUDIES ON NUT CONSUMPTION AND SERUM LIPID CHANGES

A total of 13 published studies to date have been identified in the literature examining the relationship between specific nut consumption and serum lipid changes. All were published in the last 10 years. All of these studies have been reviewed and summarized and some of their characteristics and results are shown in Tables 28.4, 28.5, and 28.6.

Differences exist among the studies represented: studies were conducted in various countries around the world, and for differing lengths of time, between 2 weeks to 6 months. The most-studied nuts have been almonds[17–19,21,25] and walnuts.[17,20,21,24,29] Some research has been done on peanuts,[23,28]macadamia nuts,[22] hazelnuts,[29] and pistachios.[26] The study designs varied from crossover,[17,18,20,22,24,26,27,29] parallel arm,[23,25] and pre–post or consecutive.[19,21,28] The administration of diets and the rigor of control varied from well-controlled metabolic-type feeding studies,[17,18,20,27] to strong dietary advice and dosage of nuts to free-living individuals,[21,25,29] to less rigid dietary advice and overall control.[19,22–24,26,28] Diverse groups of either healthy volunteers or outpatient clinic subjects that varied in age, ethnicity, gender, and baseline serum cholesterol levels (normal or hypercholesterolemic) were studied. Most studies used whole nuts with a wide range of intake, between 35 and 110 g/day during the dietary interventions.

Two studies used nuts along with other foods of similar fatty acid profile primarily to compare the direct effects of monounsaturated fatty acids (MUFA) and PFA on blood lipids and lipoproteins.[17,18] The rest of the studies compared the effects of a nut diet against a relatively low-fat/high-carbohydrate diet,[20,22–24,29] against a high-saturated-fat diet,[21,25,27] or against the subjects' usual diet.[19,26,28,29]

Although the variables and intakes differed between the studies, all showed a favorable effect on serum lipid values, or being hypocholesterolemic, generally with a reduction in total cholesterol and LDL-cholesterol levels. In some studies, the nut diets decreased HDL-cholesterol levels,[17,20,26] while in others showed an increase,[22,24,26,27] although these changes were generally not statistically significant. Triacylglyceride levels had a tendency to decrease but did not achieve statistical significance in most studies. The apparent inconsistency in results on HDL and triacylglycerides could be partially explained by the different designs and composition of the reference diet.

Although differing in methodology and dietary control, collectively these investigations provide substantial evidence that nut consumption has a hypocholesterolemic effect and is therefore protective against CHD by lessening the serum lipid–related risk factors. These effects were seen over a wide range of studies with various geographic groups, gender, ages, races, dietary regimens, and baseline lipid levels.

Kris-Etherton and colleagues[30] conducted an analysis comparing the observed changes in total, LDL, and HDL cholesterol on five of the above human nutrition studies[20–24] with predicted changes by cholesterol-lowering equations.[31–33] These equations factor the effect of dietary saturated, monounsaturated, and polyunsaturated fatty acids on serum cholesterol level outcomes. A graphic display of this comparison is shown in Figure 28.4. Collectively, the magnitude of the cholesterol-lowering effect from the nut diets is about 25% greater than was predicted by the equations. Thus, this analysis suggests that other nonfatty components of nuts may have a cholesterol-lowering effect as well.

TABLE 28.4
Summary of Published Nutrition Studies — Designs and Characteristics

Ref.	Type of Nut	Where Conducted	Subjects	Baseline Mean Serum Cholesterol	Study Design	Length of Diet Periods
17	Almonds and walnuts	Israel	22 males	147 mg/dl (3.81 mmol/l)	Randomized, controlled, crossover feeding trial	Two 12-week dietary periods
18	Almonds	Israel	17 males	156 mg/dl (4.03 mmol/l)	Randomized, controlled, crossover feeding trial	Two 12-week dietary periods
19	Almonds	California	13 males / 13 females, hypercholesterolemic	235 mg/dl (6.08 mmol/l)	Dietary advice, pre-post supplemental field study	9 weeks
20	Walnuts	California	18 males	198 mg/dl (5.12 mmol/l)	Randomized, controlled crossover feeding trial	Two 4-week dietary periods
21	Almonds and walnuts	Australia	16 males	199 mg/dl (5.15 mmol/l)	Dietary advice, consecutive supplemental field study	Three, 3-week dietary periods
22	Macadamia	Australia	7 males / 7 females, hypercholesterolemic	220 mg/dl (5.69 mmol/l)	Dietary advice, randomized, crossover field study	Two 4-week dietary periods
23	Peanuts	Florida	25 females, post-menopausal, hypercholesterolemic	255 mg/dl (6.60 mmol/l)	Dietary advice, parallel arm field study	6 months
24	Walnuts	New Zealand	21 males, hypercholesterolemic	254 mg/dl (6.58 mmol/l)	Dietary advice, randomized, crossover clinical study	Two 4-week dietary periods
25	Almonds	California	30 males and females, hypercholesterolemic	251 mg/dl (6.49 mmol/l)	Dietary advice, parallel arm field study	4 weeks
26	Pistachios	California	4 males / 6 females, hypercholesterolemic	243 mg/dl (6.28 mmol/l)	Dietary advice, randomized crossover clinical study	Two 3-week dietary periods
27	Peanuts	Pennsylvania	9 males / 13 females	188 mg/dl (4.88 mmol/l)	Randomized, controlled crossover, feeding trial	Three 24-day dietary periods
28	Hazelnuts	Turkey	18 males / 12 females, adolescents	132 mg/dl (3.41 mmol/l)	Dietary advice, pre-post supplemental field study	30 days
29	Walnuts	Spain	28 males / 27 females, hypercholesterolemic	278 mg/dl (7.21 mmol/l)	Dietary advice, randomized, crossover clinical study	Two 6-week dietary periods

TABLE 28.5
Summary of Published Nutrition Studies — Dietary Characteristics

Ref.	Diet Descriptions	Total Fat[a]	SFA[a]	MUFA[a]	PUFA[a]	Cholesterol (mg)
17	High MUFA with almonds, olive oil, and avocado	33	8	16	7	300
	High PUFA with walnuts, safflower, and soy oils	31	7	6	16	300
18	High MUFA with almonds (≈100 g/day), olive oil and avocado	33	7	17	8	308
	Low fat, high carbohydrate	18	5	7	6	328
19	High MUFA with almonds (100 g/day) and olive oil	37	5	25	7	134
	Low fat, usual diet	29	6	1	6	184
20	High PUFA with walnuts (84 g/day)	31	6	7	17	125
	NCEP Step I diet	29	9	9	10	237
21	High MUFA with almonds (84 g/day)	36	8	17	8	199
	High PUFA with walnuts (68 g/day)	37	9	10	16	235
	High SFA with 50 g/day peanuts, 40 g/day coconut, and 50 g/day confectionery	36	16	12	7	205
22	High MUFA with macadamia nuts	42	11	27	4	190
	Low fat, high carbohydrate	21	9	8	5	218
23	High MUFA, low fat with peanuts (35–68 g/day)	26	5	14	2	130
	Low fat diet	17	5	6	2	129
24	High PUFA with walnuts (78 g/day)	38	10	10	16	230
	Low fat diet	30	12	10	5	320
25	High MUFA with almonds (100 g/day) and almond oil	40	5	28	7	72
	High SFA with cheese and butter	35	17	15	3	269
26	Pistachio (20% energy) diet	39[b]	8[b]	15[b]	6[b]	NR
	Usual diet	37[b]	11[b]	11[b]	4[b]	NR
27	High MUFA with peanuts and peanut butter	36	8	18	10	200
	NCEP Step II, low fat diet	25	7	12	6	200
	High SFA (average American) diet	34	16	11	7	200
28	High MUFA with hazelnuts (≈ 68 g/day)	NR	NR	NR	NR	NR
29	Step I with walnuts (41–56 g/day)	33	6	13	12	253
	Step I (Mediterranean) diet	31	7	17	5	264

Abbreviations: SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids; NCEP = National Cholesterol Education Program; NR = not reported

[a] Expressed as a percentage of energy.

[b] Reported as median values.

TABLE 28.6
Summary of Published Nutrition Studies — Serum Lipid Changes[a]

Ref.	Diet Comparisons	Total Cholesterol	LDL Cholesterol	HDL Cholesterol	Triacylglycerol
17	High MUFA vs. baseline diet	↓10	↓14	0 (NS)	↓10 (NS)
	High PUFA vs. baseline diet	↓16	↓20	↓4 (NS)	↓9 (NS)
18	High MUFA vs. baseline diet	↓8	↓14	0 (NS)	↓4 (NS)
19	Almond diet vs. usual diet	↓9	↓12	0 (NS)	↓4 (NS)
20	Walnut diet vs. NCEP Step I diet	↓12	↓16	↓5	↓8 (NS)
21	Almond diet vs. high SFA	↓7	↓10	0 (NS)	↑1 (NS)
	Walnut diet vs. high SFA	↓5	↓9	↑3 (NS)	↑4 (NS)
22	Macadamia diet vs. usual diet	↓8	↓11	↑1 (NS)	↓21
	Low-fat, high-carbohydrate diet vs. usual diet	↓8	↓11	↓13	↓9 (NS)
23	Peanut diet vs. usual diet	↓10	↓12	0 (NS)	↓1 (NS)
	Low-fat diet vs. usual diet	↓2 (NS)	0 (NS)	0 (NS)	↑12 (NS)
24	Walnut diet vs. low fat diet	↓2 (NS)	↓4 (NS)	↑3 (NS)	↑8 (NS)
25	Almond diet vs. usual diet	↓12	↓16	0 (NS)	↓7
	High SFA vs. usual diet	↑4	↑5	↑7 (NS)	↓7
26	Pistachio diet vs. usual diet	↓4[a]	↓6 (NS)[a]	↑8 (NS)[a]	↓5 (NS)[a]
27	Peanut diet vs. high-SFA diet	↓11	↓14	↓2 (NS)	↓13
	Peanut diet vs. NCEP Step II diet	↓2	(NS)	↓1 (NS)	↓21
28	Hazelnut diet vs. usual diet	↓6	↓18	↑7	↑24
29	Walnut diet vs. Step I (Mediterranean) Diet	↓4	↓6	↑3 (NS)	↓6 (NS)
	Walnut diet vs. baseline diet	↓9	↓11	↓2 (NS)	↓8

[a] Values expressed in percent (%) change, ↓ denotes decrease, ↑ denotes increase. Unless otherwise indicated all changes are statistically significant. (NS) denotes non significant, p value > 0.05. NR denotes not reported.

[b] Reported as median values.

TABLE 28.7
Nutrient and Non-Nutrient Constituents in Nuts, and Possible Mechanistic Action on CHD

Constituent	Mechanism	Ref.
High in unsaturated fats: monounsaturated and polyunsaturated	Lowers total serum cholesterol and improves lipoprotein profile	17–29
Nut proteins:		
high in arginine amino acid	Precursor of nitric oxide	36–39
low in lysine amino acid	(endothelium-derived relaxing factor)	41–42
Dietary fiber	Lowers serum LDL cholesterol levels	
Tocopherols	Determine oxidative resistance of LDL	46–47
Folic acid	Decreases blood homocysteine levels	54
Magnesium	Ventricle arrhythmia	56
Copper	Deficiency is associated with increased blood cholesterol, blood pressure	57
Phytochemicals: flavanoids, phenolic compounds (ellagic acid), luteolin, tocotrienols, isoflavone, saponins, plant sterols, tannins	Antioxidant potential (?)	30

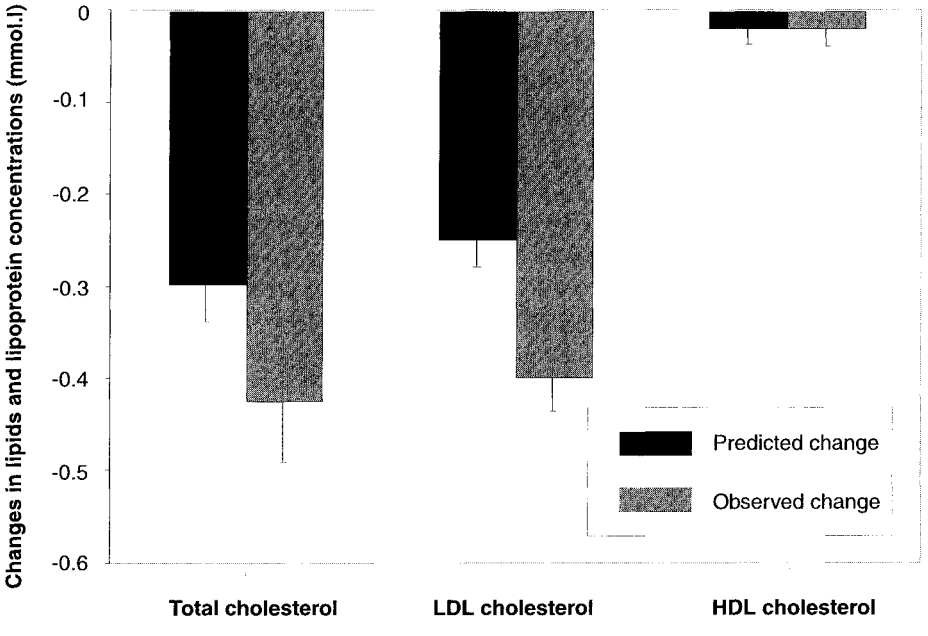

FIGURE 28.4 Comparison of observed changes in total, LDL, and HDL cholesterol concentrations with those calculated by using predictive equations for plasma cholesterol.

VI. MECHANISM OF ACTION

The improvement of serum lipid profiles is now a well-substantiated mechanism by which nut consumption reduces the risk of CHD. However, changes in blood lipids alone do not explain the magnitude of the CHD risk reduction of approximately 40 to 50% found in the epidemiological

studies noted above.[9–11] It has been shown that for every 1% reduction in LDL cholesterol there is a ~1.5% reduction in the incidence of CHD.[34] From the nutrition studies summarized in Table 28.6, it can be observed that the nut diets obtained a maximum decrease of 16% in LDL cholesterol; thus, the estimated decrease in CHD would be 24%. Using the epidemiological estimate leaves ~15% or more decrease in CHD risk unaccounted for, suggesting that other unidentified mechanisms beyond serum lipids might contribute to the cardioprotective effects of nuts.

This section reviews the potential mechanisms that nut constituents may exert on CHD risk reduction beyond changes in serum lipid levels. Table 28.7 lists nutrient and nonnutrient constituents in nuts and their suggested antiatherogenic mechanisms of action.

Nuts afford a substantial amount of plant protein ranging from 14 to 26% by weight. Compared with animal protein, plant protein elicits better effects on serum lipids. A meta-analysis of human studies on soy protein showed hypocholesterolemic effects of this protein.[35] Nut protein has a similar amino acid profile to soy protein, and it is possible that it could share similar effects, although no studies have yet examined this.

Nut protein is high in arginine, and has a high arginine:lysine ratio, making it one of the lowest among high-protein foods (see Table 28.2). Arginine may play a role in providing a possible antiatherogenic effect. Recent research has shown that arginine is the precursor of nitric oxide, a potent endogenous vasodilator. This endothelium-derived relaxing factor has demonstrated the following actions: induces relaxation of vascular smooth muscle, inhibits platelet aggregation, monocyte adherence, chemotaxis, and vascular smooth muscle cell proliferation.[36–39]An antiatherogenic effect of a low lysine:arginine ratio in dietary protein has been postulated in animals[40] and humans.[41] In several animal studies, investigators have found a direct relationship of this dietary amino acid ratio to levels of serum cholesterol.[42,43]

Walnuts are different from most nuts in their fatty acid composition. Walnut fat is primarily polyunsaturated fatty acids (PUFA). They contain appreciable amounts of n-3 essential fatty acids, i.e., α-linolenic acid, which have probable cardioprotective effects. In the Health Professionals Study, an inverse relationship between α-linolenic acid in the diet and risk for myocardial infarction was shown.[44]

The dietary fiber content of nuts is high, ranging from 4 to 11% by weight, and consist of as much as ~25% soluble fiber. The effects of soluble fiber in lowering total and LDL cholesterol are well recognized.

Nuts are an excellent dietary source of tocopherols, with more vitamin E than any other food group, apart from extracted vegetable oils. Almonds and hazelnuts are particularly rich in α-tocopherol, whereas walnuts and pecans are high in γ-tocopherols. Vitamin E in high doses has been recognized in various studies[45,46] to exhibit an independent protective effect on CHD in men and women, although this finding is still controversial. As mentioned above in the Iowa Women's Health Study, statistical adjustments for α-tocopherol reduced the protective effect of nuts by 12%, thereby implicating it as a mediating factor in the nut–CHD relationship.

Antioxidant activity is a basic function of vitamin E: trapping free radicals and thus preventing oxidation of unsaturated fatty acids in tissues. The content of α-tocopherol has been shown to determine the oxidative resistance of LDL particles.[46–47] α-Tocopherol is transported within LDL and HDL particles and consequently may offer insight into the oxidative process that can affect unsaturated fatty acids as well as LDL-cholesterol.

The oxidation of LDL particles may influence its atherogenic potential, as it is implicated in foam cell accumulation, has been shown to be cytotoxic, and is more atherogenic than normal LDL-cholesterol.[48] The susceptibility to oxidation was found to be lower on high-MUFA-containing diets that displaced high-PUFA-containing diets.[49–52] However, two studies showed that PUFA from walnuts did not increase the susceptibility of LDL particles to oxidation compared with a high-MUFA/Mediterranean diet,[29,52] or a Step I diet.[53] A decreased tendency of lipid peroxidation was shown in a study incorporating 100 g of almond into the daily diet.[17]

Folic acid in nuts may decrease blood homocysteine levels, which has been linked to CHD risk.[54] Elevated homocysteine concentration has been shown in epidemiological studies to be a risk factor for several vascular diseases, and has been correlated with the severity of carotid artery stenosis.[55]

Most nuts contain a good portion of magnesium, approximately 8 to 20% of the Daily Recommended Intake in a 1-oz serving. Low magnesium is related to dysrhythmias, myocardial infarction, muscle relaxation, and possibly hypertension.[56] On average, 1 oz of nuts contains approximately 18% of the Daily Recommended Intake for copper and therefore can be considered an excellent source of this mineral. Copper plays a key role in hematopoiesis and diets low in copper have been associated with adverse changes in serum lipids and blood pressure.[57]

Nuts, as in other plant foods, are a source of phytochemicals. Only a few thus far have been identified in nuts: flavonoids, phenolic compounds, luteolin (a major antioxidant), tocotrienols, isoflavones, ellagic acid, as well as other components like plant sterols.[58] Flavonoids, a subset of antioxidant plant polyphenols, have been shown to be inversely related to CHD mortality.[30] Sterols from other plant sources have been shown to inhibit cholesterol absorption.

VII. ARE NUTS A FUNCTIONAL FOOD?

It is generally understood that functional foods are those that have health benefits beyond the nutrients they contain.[59] As was presented throughout this chapter there are compelling reasons for considering the inclusion of nuts among listed functional foods. First, nut consumption has been found to decrease the risk of CHD. This consistent health outcome demonstrated in several epidemiological studies of chronic diseases is uncommon among other foods classified as functional. Second, nuts seem to lower total and LDL serum cholesterol in all human nutrition studies that have investigated these health outcomes. Additionally, this cholesterol-lowering effect is greater than that estimated by their fatty acid composition. Third, the magnitude of the CHD risk reduction (approximately 50%) is greater than might be anticipated by changes in blood lipids alone.

Thus, based on these lines of reasoning, it is proposed that nuts be considered functional foods. Contrary to other plant foods, there is very little known beyond the nutritional components in nuts. As more data become available on phytochemicals and other non-nutrient components present in nuts, the above points might be clarified.

VIII. FUTURE RESEARCH DIRECTIONS

Future research should include human feeding studies with longer dietary periods to see if the short-term findings can be duplicated in the long term. Research to identify non-nutrient components such as phytochemicals is gaining momentum in many plant foods. Unfortunately, the focus has not been as strong for the non-nutrients in nuts. More data are needed to generate new hypotheses.

Currently, there is interest in investigating if diets high in unsaturated fats have some weight reduction effect as compared with high-saturated-fat diets. It could be possible that high MUFA/PUFA contents in nuts increases satiety, leading to less caloric intake. Several studies have shown that despite an increase of energy from nuts, no significant change in body weight was found.[60]

IX. CONCLUSION

In summary, epidemiological studies supported by human nutrition studies have established that nuts, along with fruits, vegetables, legumes, and whole grains, are a component of healthy diets. Longitudinal studies showed a strong reduced risk of fatal and nonfatal CHD, and a possible reduction in all-cause mortality. Human nutrition studies provided consistent findings for nuts favorably modulating blood lipid levels, as seen in a reduction of total serum and LDL cholesterol,

without significantly modifying HDL cholesterol. These findings warrant the consideration of nuts as a recognized functional food, and add legitimacy for recommending the regular inclusion of nuts as part of a health-promoting diet.

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29 Colon Cancer: Dietary Fiber and Beyond

Gene A. Spiller and Monica Spiller

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I. INTRODUCTION

The possible role of dietary plant fiber in the prevention of colonic diseases, including colon cancer — a major cancer in industrialized countries — emerged in the late 1960s and early 1970s from the research of Walker[1] in South Africa, Burkitt and Trowell[2] in Uganda, and the writing of Cleave in Britain.[3] Their work reported a low incidence of colonic diseases in the African natives, consuming large amounts of unrefined plant foods and small amounts of animal foods, and a high incidence of the disease in the European residents who consumed high-meat, refined-carbohydrate diets, low in plant foods. Other early studies supporting a preventive effect of high-fiber diets on colon cancer included those of Malhotra[4] in India who found a low incidence of colon cancer in northern India, where the diet contains large amounts of dietary fiber, and a high incidence of colon cancer in southern India, where the diet is low in dietary fiber.

As both Burkitt and Trowell were well known to the medical community, their work found wider acceptance than that of lesser-known authors when they published various papers in the late 1960s and early 1970s.

Both animal and case-control studies support the protective role in colon cancer prevention of fiber or of fiber-rich foods. Epidemiological studies are not as supportive for reasons that need to be carefully considered and that will be discussed later in this chapter.

II. ANIMAL STUDIES

Erschoff, a pioneer in the field of studying protective effects of dietary fiber in laboratory animal models treated with various carcinogens, demonstrated in the 1950s and 1960s that many otherwise toxic levels of various substances become either less toxic or nontoxic when fed together with foods high in dietary fiber or some of the fiber polymers.[5] Later, many animal studies in the 1970s and 1980s showed that higher intake of some purified fibers or foods high in fiber such as wheat

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bran protected experimental animals from colonic tumors after feeding chemical carcinogens. A protective effect was shown by numerous investigators for cellulose, wheat bran, and alfalfa. No effect was found for some soluble fiber not part of normal foods, such as guar gum.[5]

In several rat studies wheat bran was shown protective against carcinogens such as 1,2-dimethylhydrazine, azoxymethane, 3,2-dimethyl-4-aminobiphenyl; citrus fiber against azoxymethane and 3,2-dimethyl-4-aminobiphenyl; and alfalfa against azoxymethane. Cellulose showed protective effect against 1,2-dimethylhydrazine and both cellulose and pectin against azoxymethane.[5]

III. HUMAN STUDIES

Over the past three decades numerous studies have examined the relationship between dietary fiber intake and colon cancer with mixed results. The Howe and colleagues[6] meta-analysis of 13 intercountry, case-control studies reported an inverse relationship in 12 of the 13 studies between risk of colorectal cancer and fiber intake. Their data indicated an inverse dose–response relationship between fiber and colon cancer risk. Compared with subjects in the highest quintile of fiber intake, subjects in the lowest quintile (30 g/day vs. 10 g/day) were found to have twice the risk. Since then, several reports of other case-control studies of colon cancer and fiber have been reported. Meyer and White[7] found that compared with population-based controls, subjects without colon cancer consumed higher amounts of fiber than those with colon cancer. In another multicenter case-control study by Slatter and colleagues,[8] colon cancer risk was found to be higher with a low intake of fiber. Although not all results of case-control studies agree, overall they provide convincing support for an inverse association between risk of colon cancer and dietary fiber intake.

Findings from epidemiological studies have been more equivocal with regard to the protective effects of fiber.[9,10] For instance, Giovannucci and colleagues[11] found no clear association between fiber or vegetable intake in their observation study based on quintiles that ranged from 13 to 34 g/day. The large Nurses' Health Study[12] also reported no significant association between fiber intake and risk of colorectal adenomas, based on quintiles of fiber intake that ranged from a low of about 9 g/day to a high of 29 g/day. In these two not truly high-fiber diets were consumed by the population studied. On the other hand, the Thun and colleagues[13] prospective epidemiological investigation reported a protective effect on colon cancer with more frequent consumption of vegetables and high-fiber grains, and the cross-sectional data provided by Frazer[14] for the California Seventh-Day Adventists Study supported a lowered risk of colon cancer in vegetarians, who often consume more dietary fiber compared with nonvegetarian subjects.

IV. REFINED FOOD STUDIES

Further support for the role of high-fiber foods in cancer protection came in 1999 from a study by Chatenoud and colleagues,[15] who studied groups of hospital patients in Milan, Italy and found that there was a consistent pattern of increasing cancer risk with increasing intake of refined cereals. Several common neoplasms, including those of the digestive tract, the larynx, and the thyroid, showed augmented occurrence among those patients who reported the highest intake of refined bread, pasta, and rice, which are very common in the Italian diet.

V. THE PHYTOCHEMICAL LINK

In the 1990s compounds previously considered of little or no value in health maintenance and disease prevention began to find their proper place in nutrition. These compounds, which became know as phytochemicals, are present in high-fiber plant foods and they may work together with dietary fiber in colon cancer prevention. Typical of these phytochemicals are tartaric acid in grapes and raisins, an acid that affects colon function; phytic acid in whole grains; bioflavonoids in oranges;

and various tocopherols in nuts. Another large group of compounds often linked to fiber are the many powerful polyphenolic antioxidants such as quercetin in fresh and dried fruits. Studies on fiber consumption need to consider the type, amount, and level of phytochemicals present.

VI. MECHANISM OF ACTION OF DIETARY FIBER

There have been many hypotheses proposed for the protective effect of dietary fiber. The most widely accepted is that dietary fiber decreases intestinal transit time and increases fecal bulk. The faster transit time expedites removal of both ingested and endogenous carcinogens, stagnant in a constipated colon. The increased fecal bulk dilutes the carcinogens.

Some fibers and tartaric acid alter the pattern of primary and secondary bile acids in intestines in manner favorable to protection. In addition, the digestion of fiber by colonic bacteria produces short-chain fatty acid such as acetic and propionic that lower fecal pH, considered by some investigators more protective than a more alkaline pH.

VIII. CONCLUSION

The conflicting results in epidemiological studies may be due to one or more of the following problems:

- The first problem is the attempt to consider dietary fiber in isolation from both the other components of the diet and the associated phytochemicals.
- The second problem is that dietary fiber is a very bulky substance: while the addition or subtraction of a vitamin or mineral hardly affects the consumption of other foods, a diet truly high in dietary fiber is also high in unrefined plants foods from seeds to vegetables and fruits. This means that the diet has to be lower in animal products. The Seventh-Day Adventist Study is an example of just such a situation.
- The third problem is that the phytochemicals that are associated with fiber are higher in a diet high in whole, unrefined plant foods. This causes another major problem for the investigator who wants to pinpoint a single factor, a problem similar to the displacement by study foods of foods normally consumed in the diet.
- The fourth problem is that some studies, like the Fuchs study,[12] consider 25 to 35 g of dietary fiber an amount that justifies the conclusion that dietary fiber is not protective. Even though there is no hard data, certainly the daily intake of fiber by rural South Africans and Ugandans had to be much higher.
- The fifth major problem is that the definition of dietary fiber may be too simplistic. Dietary fiber is extremely complex, defies isolation without damage, and simply considering soluble vs. insoluble fiber is not sufficient to overcome this problem. The fibers of wheat, that of raisins and figs, that of carrot, and of all other plant foods are complex entities that go beyond the two dietary fiber subfractions now called soluble and insoluble fiber.
- The sixth problem is processing and preparation. Was the fiber raw or cooked? No consideration has been given to this condition, something which so powerfully affects the physical status of the fiber, something that is evident to anyone consuming a food raw or cooked.

A careful review of the many studies shows that the Burkitt–Trowell hypothesis that stated that diets *truly high* in unrefined plants foods and in fiber are protective against colon cancer is valid, but much work is ahead in fiber definitions and in studying the role of methods of preparation of the food and the role of associated substances like antioxidant phytochemicals, and phytic and

tartaric acids. Perhaps an old definition should be revived, that of a "dietary fiber complex," which was proposed in the 1970s, as fiber defies a simplistic definition just as much as fats or proteins do.[16]

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30 Stability Testing of Nutraceuticals and Functional Foods

Leonard N. Bell

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I. INTRODUCTION

For any product to gain acceptance by consumers, its quality and nutritional content must be consistent from the time of processing, through distribution and storage, to the final consumption of the product. While the food and pharmaceutical industries have guidelines for determining product stability and ensuring label claims, such is often not the case with nutraceutical products. However, nutraceuticals are subjected to the same types of quality losses during storage as food and pharmaceutical products. To maintain consumer acceptance and avoid possible governmental action, nutraceuticals should be evaluated for stability, including determination of product shelf life and ensuring accurate label claims. The physiological benefits of nutraceuticals are achieved only if (1) the product is consumed and (2) the bioactive substance is present at the required concentration. If either of these conditions is not met due to a product quality change during storage, the nutraceutical or functional food loses its beneficial effect.

The length of time after processing that a product remains acceptable to the consumer from a quality or safety perspective is known as the product shelf life. A product can lose shelf life in several ways. Microbial growth in a product can decrease sensory acceptability via spoilage or provide a health risk. Physical changes, such as hardening of dried fruit and softening of crunchy cereals, are other mechanisms of shelf life loss. Finally, chemical reactions can occur during processing and storage, resulting in quality changes such as unacceptable color development, nutrient loss, and flavor modification.

The Food and Drug Administration regulates food and pharmaceutical products with respect to nutritional and potency values placed on labels; these values can decrease over time due to deteriorative chemical reactions. A nonfortified food product, such as pure orange juice, is expected

0-8493-8734-5/01/$0.00+$.50
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to contain at least 80% of the label value for a listed nutrient (e.g., vitamin C), while fortified foods, such as orange juice with extra vitamin C, must contain 100% of the label value for the fortified nutrient, in this case vitamin C.[1] If the nutrient degrades over time and the amount of the nutrient falls below that required based on the food type and label value, the product is considered misbranded by the Food, Drug, and Cosmetic Act and becomes illegal.[1] For pharmaceutical products, the expiration date is determined by the intersection of two lines: 90% of the label potency value and the lower 95% confidence limit of concentration decrease as a function of time.[2–4] Labeling issues with nutraceuticals and dietary supplements are less clear because little is known about their chemical stability and, for some products, the active ingredient has not been identified. Regulation of products is quite limited and was not helped by the recent passage of the Dietary Supplement Health and Education Act (DSHEA).[5,6] However, some nutraceuticals are in the form of a "food" and as such are subject to the same regulations as traditional foods, which is one reason understanding stability issues associated with the product is important.

Nutraceuticals and functional foods include a vast array of different product types, containing a variety of different biologically active chemical compounds. Some of these compounds include isoflavones, flavonoids, carotenoids, bioactive peptides, and vitamins. Nutraceuticals can exist as dry powders, intermediate-moisture products (e.g., bars), and beverages. Some type of food processing (e.g., mixing, heating, drying) is required to prepare the aforementioned product types. Nutraceutical producers need to know how much biologically active ingredient must be consumed to obtain the desired physiological effect and compare this value with the amount of the ingredient remaining in the product after processing, distribution, and storage. Dry powders are often stored for over a year; however, the consumer may or may not be obtaining the desired nutraceutical "dose" from this product. For example, the ginsenoside contents in ginseng preparations were found to vary from close to 0 to as high as 9%.[6,7] While some of the product inconsistency is due to variations of species, maturity, and plant source (e.g., root vs. leaf),[8] the potential for degradative reactions persists and product stability should be evaluated.

To address the issues raised previously, stability testing needs to be performed on the product. The first step of the process is to identify the bioactive substance in the nutraceutical. Coffee contains hundreds of chemical compounds, but caffeine is the well-known bioactive compound, providing the stimulatory effect. Similarly, hypericin is the active substance found in St. John's wort.[9,10] After the bioactive compound has been identified, an accurate and precise analytical method for its determination is required to measure its concentration as a function of time under different conditions, including temperature, moisture, oxygen, and added ingredients. After the concentration of the active compound is measured over time, the data undergo kinetic modeling and shelf life predictions can be made for conditions typical of product distribution and storage. The basic aspects of stability testing are presented subsequently.

II. KINETIC MODELING OF CHEMICAL REACTIONS

Assuming the biologically active substance has been identified and a precise analytical method for its detection developed, the first step of shelf life testing is to collect concentration data as a function of time at a constant temperature. The greater the number of data points along with the greater extent of concentration change, the more statistically valid the mathematical modeling becomes.[11] It is most desirable to have at least 13 data points and to collect data past a 50% decrease in ingredient concentration because this allows for reasonable confidence limits to be calculated.[12] However, typically 10 data points are equally effective with only a slight increase in the confidence limits as compared to 13 data points. Once the number of data points is reduced below 8, the confidence limits become significantly larger and shelf life predictions become increasingly error prone.

Chemical reactions consist of reactants being converted into products as outlined below.

$$aA \rightarrow bB \tag{30.1}$$

The loss of reactant A or the accumulation of product B results in the product losing shelf life. The elementary reaction above proceeds at a specific rate (concentration change vs. time, $-d[A]/dt$); the rate expression is defined as:

$$-d[A]/(a)dt = +d[B]/(b)dt = k[A]^a = k[B]^b \tag{30.2}$$

where $[A]$ and $[B]$ are concentrations of reactant and product, respectively; a and b are molecularities of the compounds; and k is the rate constant.[13] The molecularity represents the true number of molecules involved in the reaction as determined from its mechanism; thus, for complex chemical reactions, the true rate expression cannot be determined from the overall reaction pathway.[13] Rate constants depend upon temperature and their determination is central for shelf life predictions.

In complex food and pharmaceutical systems, many variables influence chemical reactions, and the overall pathway for a given reaction can be written as

$$\text{reactant(s)} \rightarrow \text{product(s)} \tag{30.3}$$

where the true reaction mechanism is generally ignored. For this type of reaction, the rate expression for reactant loss is written as

$$-d[A]/dt = k_{obs}[A]^n \tag{30.4}$$

where $-d[A]/dt$ is the concentration change over time, $[A]$ is the reactant concentration, k_{obs} is the observed or apparent rate constant, and n is the reaction order. As mentioned, for many chemical reactions in foods and pharmaceuticals, n is not the true reaction order as determined from physical chemistry, but is the pseudo-order of the reaction.

The reaction order can be determined from several techniques, as discussed in physical chemistry textbooks.[13] Other methods can also be found in the literature.[14,15] Because shelf life predictions are most effective with linear models, simple mathematical techniques consistent with reaction kinetic concepts are frequently employed to linearize the quality loss data. For food systems, most data can be fit to either pseudo-zero-order or pseudo-first-order kinetic models.[12] By performing a linear regression on the reactant concentration as a function of time ($n = 0$ for pseudo-zero order) and the natural log of reactant concentration as a function of time ($n = 1$ for pseudo-first order), the more appropriate order can be selected by evaluating the coefficient of determination (r^2). If an adequate number of data points have been collected past a concentration change of 50%, then the data having the highest r^2 value are usually the best pseudo-order for modeling quality loss associated with the change in reactant or product concentration.[12] Figure 30.1 shows an example of determining the pseudo-order for aspartame degradation using previously collected data,[16] where the data are plotted as both pseudo-zero order and pseudo-first order. As shown in this figure, the pseudo-first-order model provides the better linear fit and would be used to calculate the rate constant. A discussion of different pseudo-order kinetic models for calculating rate constants follows. Again, the determination of rate constants under different conditions is a critical component of shelf life studies.

The pseudo-zero-order kinetic equation ($n = 0$) for reactant loss takes the form of

$$-d[A]/dt = k_{obs}[A]^0 = k_{obs} \tag{30.5}$$

To model product formation, the negative sign in front of $-d[A]/dt$ is simply changed to a positive sign, $+d[A]/dt$. Upon integration of Equation 30.5, the following expression is derived:

$$[A] = [A]_0 - k_{obs}t \tag{30.6}$$

where $[A]_0$ is the initial reactant concentration and $[A]$ is the concentration at time, t. The pseudo-zero-order rate constant, k_{obs}, is determined from the linear slope of a pseudo-zero-order kinetic

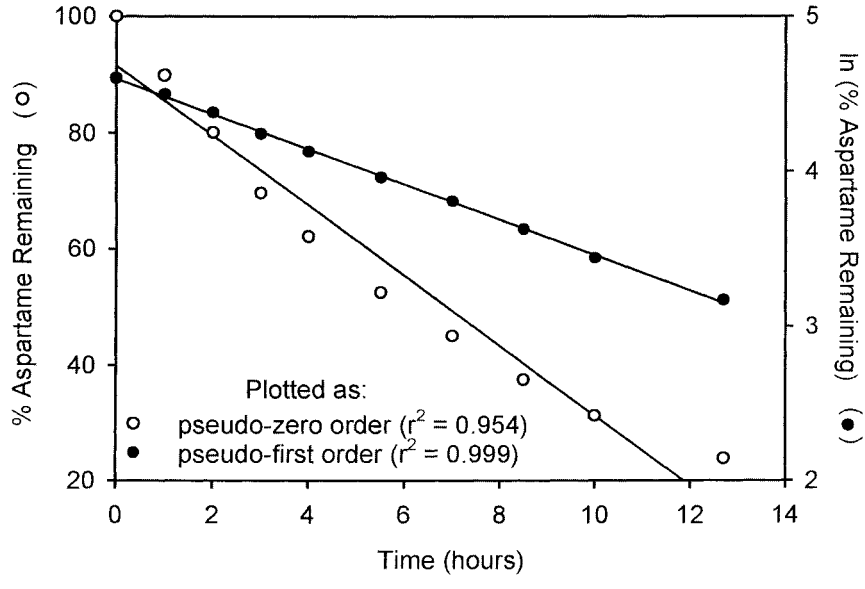

FIGURE 30.1 Aspartame degradation in 0.1 *M* phosphate buffer at pH 7 and 25°C plotted as pseudo-zero-order and pseudo-first-order kinetic models. (Data from Reference 16.)

plot, where reactant concentration, [*A*], is plotted as a function of time. Figure 30.2 shows a general example of the pseudo-zero-order kinetic plot. The time to reach 50% of the initial reactant concentration is known as the half-life. The half-life, $t_{1/2}$, of a pseudo-zero-order reaction is calculated using the following equation:

$$t_{1/2} = [A]_0/2k_{obs} \tag{30.7}$$

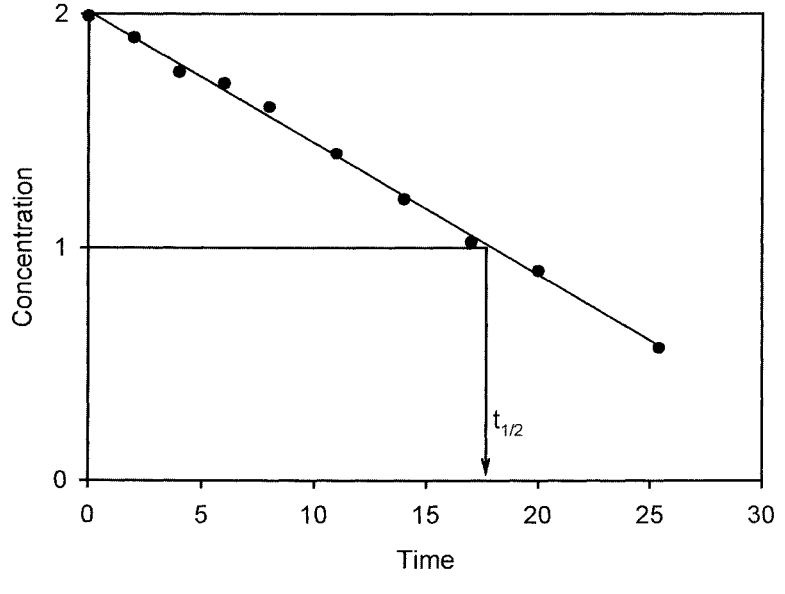

FIGURE 30.2 General example of a pseudo-zero-order kinetic plot. Slope equals pseudo-zero-order rate constant. The half-life, $t_{1/2}$, of the reaction is identified.

and is identified in Figure 30.2. The value of the half-life is dependent upon the initial reactant concentration. The formation of brown pigmentation from the nonenzymatic browning reaction is commonly modeled using pseudo-zero-order kinetics.[17–19]

The pseudo-first-order kinetic equation ($n = 1$) for reactant loss takes the form of

$$-d[A]/dt = k_{obs}[A]^1 = k_{obs}[A] \tag{30.8}$$

Again, to model product formation, the negative sign in Equation 30.8 is replaced with a positive sign. Integrating Equation 30.8, the following expression is derived:

$$\ln[A] = \ln[A]_0 - k_{obs}t \tag{30.9}$$

which can be rearranged into

$$\ln([A]/[A]_0) = -k_{obs}t \tag{30.10}$$

where $[A]_0$ is the initial reactant concentration, $[A]$ is the concentration at time, t, and $[A]/[A]_0$ is the ratio (i.e., percent) of the reactant remaining at time, t. The pseudo-first-order rate constant, k_{obs}, is determined from the linear slope of a pseudo-first-order kinetic plot, where the natural log of reactant concentration is plotted as a function of time. If log base 10 is used instead of the natural log, the slope needs to be multiplied by 2.303 to obtain the rate constant. Figure 30.3 shows a general example of the pseudo-first-order plot. The half-life, $t_{1/2}$, of a pseudo-first-order reaction is calculated as follows:

$$t_{1/2} = 0.693/k_{obs} \tag{30.11}$$

The value of the half-life is independent of the initial reactant concentration for pseudo-first order reactions, unlike pseudo-zero-order reactions. The time corresponding to the half-life is also indicated in Figure 30.3. In addition to aspartame degradation,[16,20–22] riboflavin degradation,[23]

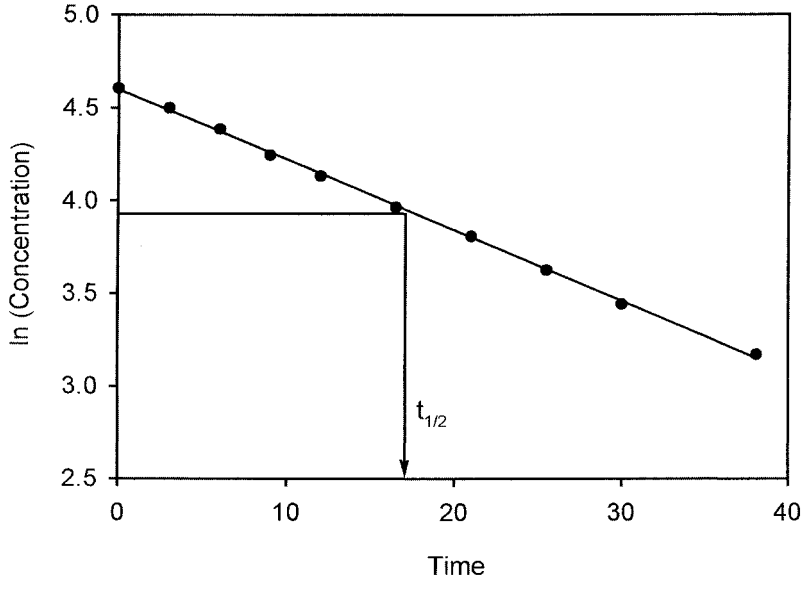

FIGURE 30.3 General example of a pseudo-first-order kinetic plot. Slope equals pseudo-first-order rate constant. The half-life, $t_{1/2}$, of the reaction is identified.

thiamin degradation,[23,24] vitamin C degradation,[25,26] and enzymatic activity loss[27] have been modeled using pseudo-first-order kinetics.

Pseudo-zero- and pseudo-first-order kinetics do not always adequately model quality loss data. When two compounds react to form a product, second-order kinetics are often more appropriate to use. An example of this is reactant loss associated with the Maillard reaction.[18,19] A discussion of second-order kinetics can be found in physical chemistry textbooks.[13] Lipid oxidation does not follow pseudo-zero-, pseudo-first-, or second-order kinetics, but has its own unique kinetic models.[28,29] The scientific literature should be consulted to determine what types of kinetic modeling have been used successfully for a particular reaction as part of a shelf life study.

A. Effect of Temperature on Stability

Temperature has a dramatic effect on the rates of chemical reaction. The primary effect of temperature is on the rate constant, although other more subtle effects also exist (e.g., changes in water activity, melting of lipids, glass–rubber transitions). Product deterioration increases as temperature increases, which is the rationale for storing food and pharmaceutical products under refrigerated conditions. By determining the effect of temperature on the rate constant of a chemical reaction, predictions can be made regarding rates at other temperatures. To evaluate this temperature effect, the pseudo-order rate constant should be determined at a minimum of three temperatures, but preferably four or five to increase the confidence of the predictions.[12]

The effect of temperature on chemical reactions occurring in food and pharmaceutical products generally follows the Arrhenius relationship:

$$k_{obs} = (A)\ \exp(-E_a/RT) \tag{30.12}$$

where A is the pre-exponential factor, E_a is the Arrhenius activation energy, R is the ideal gas constant, and T is temperature in Kelvin.[13] Using the value of 8.314 J/(mol K) for the ideal gas constant, the activation energy is in units of J/mol. A plot of the natural log of the pseudo-order rate constant as a function of the reciprocal of absolute temperature yields a straight line, as shown in Figure 30.4 for vitamin C degradation.[25] The slope and intercept of the line equal $-E_a/R$ and $\ln(A)$, respectively. Using this information, pseudo-order rate constants at any other temperature can be calculated. However, the confidence of the prediction depends upon the quality of the kinetic data, the number of temperatures used to obtain the activation energy, and the distance from the experimental temperatures one is trying to predict. Labuza and Kamman[12] have discussed aspects of optimizing the confidence of temperature extrapolations using the Arrhenius equation.

Another approach for modeling the temperature effect on chemical reactions is by calculating Q_{10} values.[12] The ratio of the rate constant at one temperature to the rate constant at 10°C lower is one definition of Q_{10}. Another definition of Q_{10} is the ratio of the shelf life at one temperature to the shelf life at a temperature 10°C higher. The Q_{10} is calculated from a shelf life plot, where the natural log of shelf life (or half-life) is plotted as a function of temperature in degrees Celsius. The Q_{10} is determined using the absolute value of the slope, b, as shown below:

$$Q_{10} = \exp(10|b|) \tag{30.13}$$

A shelf life plot of the vitamin C degradation data[25] appears in Figure 30.5. The Q_{10} value of 2.96, as determined from the slope of Figure 30.5, means that the stability of vitamin C at 25°C is approximately three times less than that at 15°C.

The relationship between the Q_{10} value and Arrhenius activation energy is shown in the following equation:[12]

$$\ln(Q_{10}) = 10E_a/RT(T + 10) \tag{30.14}$$

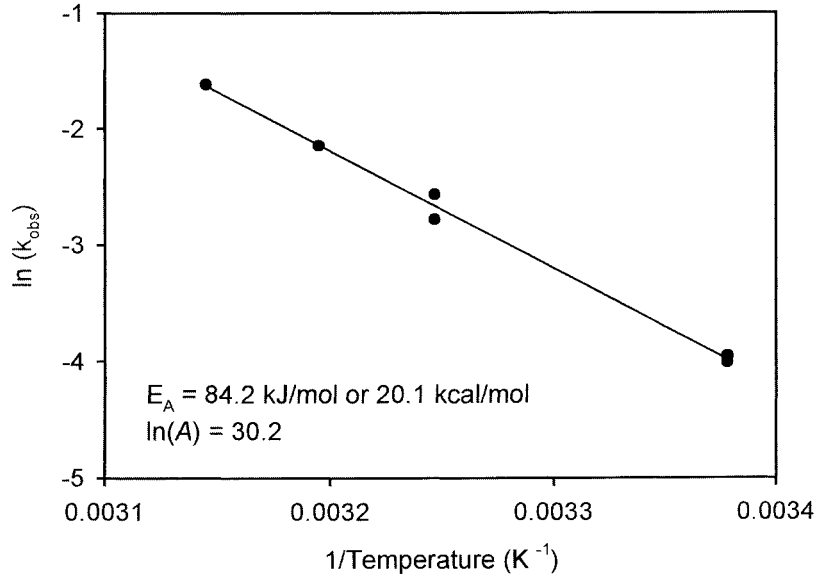

FIGURE 30.4 Arrhenius plot for vitamin C degradation in a model system at water activity 0.32. (Data from Reference 25.)

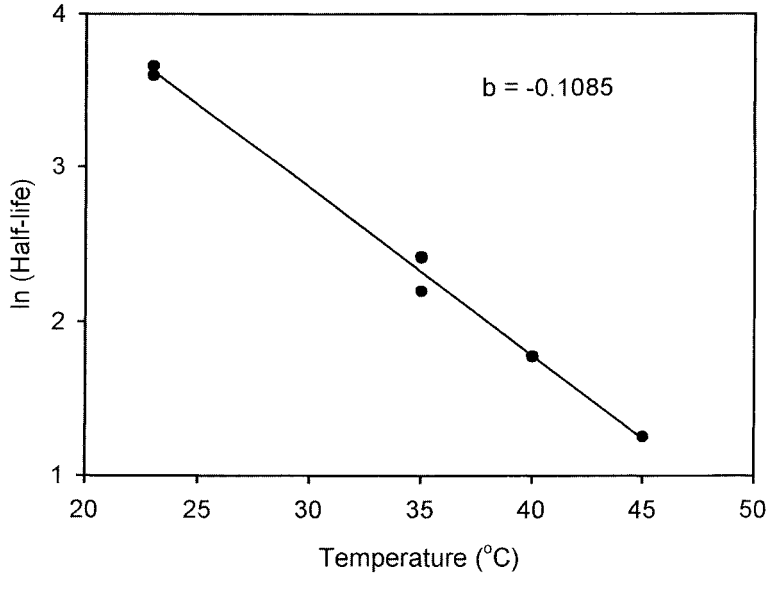

FIGURE 30.5 Shelf life plot for vitamin C degradation in a model system at water activity 0.32. (Data from Reference 25.)

This equation also shows that the Q_{10} is dependent upon temperature, and as such, extrapolations are valid only over a narrow temperature range (i.e., <25°C). The Arrhenius activation energy is not temperature dependent and should therefore be used when trying to extrapolate kinetic data over larger temperature ranges. However, in both cases the assumption is made that no physical changes of the product (i.e., lipid melting, glass transition) occur over the extrapolated temperature range.

Temperature abuse is frequently the major factor limiting product shelf life. A prime example is outdoor storage displays of aspartame-sweetened beverages by mini-markets; these canned products sit in the hot summer sun for long periods of time before purchase. The temperature abuse these products experience enhances aspartame degradation, decreasing product sweetness and acceptability. The same situation exists for products stored in automobile trunks and garages. Establishing quality control procedures during processing and distribution can help reduce excessive temperature exposure of the product. Refrigeration, although a more expensive option, can substantially extend product shelf life. Dates and handling instructions on product labels can be used to help educate consumers about the consequences of temperature abuse. One approach for indicating more accurately the thermal history of a product is by the use of time temperature indicators (TTIs). TTIs are small tags which provide a visual indication as to the net temperature exposure of a product and may improve the accuracy of shelf life dating. Concepts associated with the use of TTIs have been discussed in the literature.[30–33] Controlling temperature abuse is the primary way to increase the shelf life of nutraceuticals and functional foods and to improve the accuracy of their nutritional labeling.

B. Effect of Moisture on Stability

Moisture content and water activity have long been recognized as important for evaluating product stability and predicting shelf life, especially of dry powders and intermediate moisture solids.[34–36] Food stability generally correlates better with water activity than moisture content.[37] Water activity (a_W), or the chemical potential of water, can be defined as:

$$a_W = p/p_o \tag{30.15}$$

where p is the partial pressure of water in a product at a given temperature and p_o is the partial pressure of pure water at that same temperature.[35,37] Water activity is the governing force that dictates moisture transfer between a product and the environment until equilibrium is obtained.[38] The relationship between water activity and equilibrium moisture content of a substance is shown via moisture sorption isotherms,[38] an example of which is shown in Figure 30.6. One of the more frequently utilized mathematical models of moisture sorption data is the GAB equation, which can be written as:

$$m = m_o kCa_W/[(1 - ka_W)(1 - ka_W + kCa_W)] \tag{30.16}$$

where m is the moisture content, m_o is the monolayer moisture content, and k and C are constants representing the thermodynamics of multilayer moisture adsorption.[39] Nonlinear regression of the moisture sorption data yields values for m_o, k, and C. Of interest is the value of m_o because many chemical reactions occur only minimally at and below this moisture content.[35,39] Chemical stability of solids generally decreases as water activity increases above m_o due to an increase in reactant mobility.[35] A stability minimum often exists at an intermediate water activity. At water activities above this minimum, stability may increase due to reactant dilution reducing the probability of molecular collision and subsequent reactivity.[35] Figure 30.7 shows the effect of water activity on aspartame shelf life,[21,40] where the shelf life is defined as the time to reach 20% of the initial aspartame concentration. The shelf life minimum is at a water activity of approximately 0.8 for aspartame at pH 5 and 30°C.

From the monolayer to the stability minimum, a plot of the log base 10 of the shelf life as a function of water activity often yields a straight line.[38] From the absolute value of the slope, B, the Q_A value can be calculated using the following equation:

$$Q_A = 10^{0.11|B|} \tag{30.17}$$

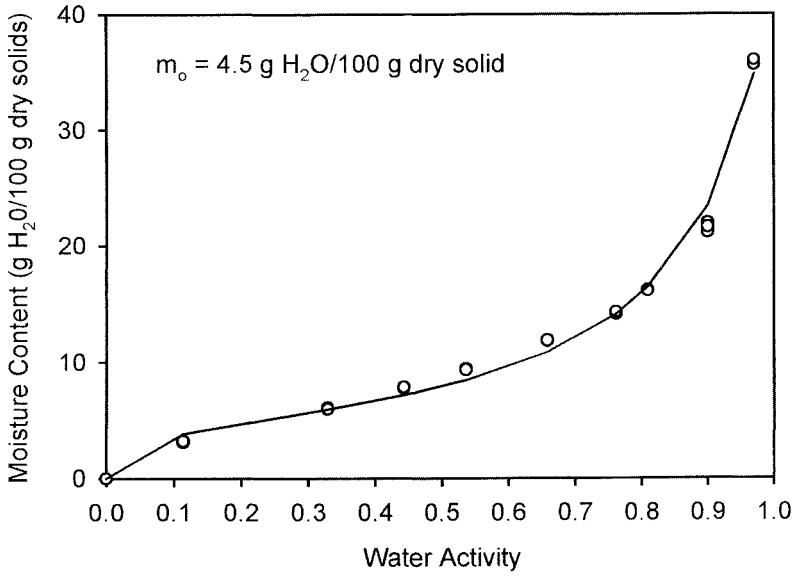

FIGURE 30.6 Moisture sorption isotherm for oat fiber at 25°C.

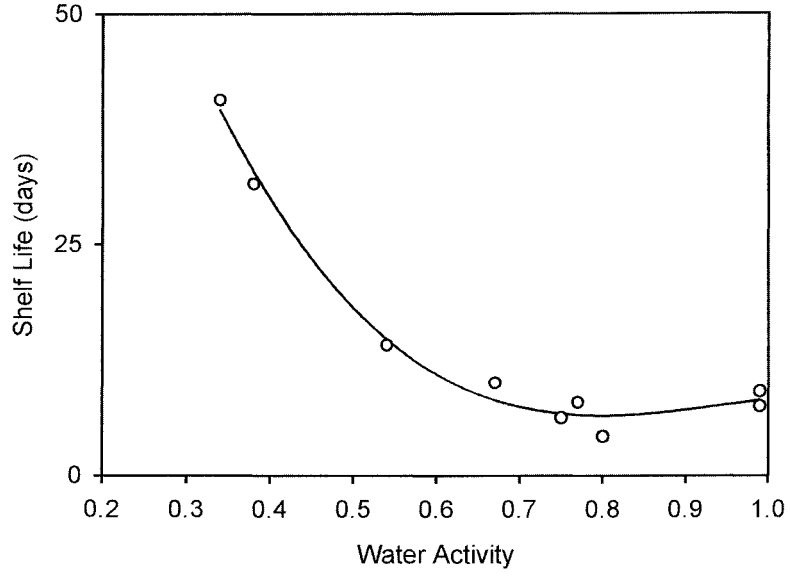

FIGURE 30.7 Shelf life of aspartame at pH 5 and 30°C as a function of water activity. (Data from References 21 and 40.)

Similar to the Q_{10} value discussed previously, the Q_A is the ratio of the shelf life at one water activity to the shelf life at a water activity 0.1 units higher. This expression can be used to predict the shelf life at different water activities within the linear range. A Q_A plot of the aspartame degradation data[21,40] is shown in Figure 30.8. From this plot, a Q_A of 1.54 was determined, meaning that if the product has a 0.1 unit increase in water activity, the shelf life decreases by 54%.

An additional factor to consider is that, in sealed packages, temperature influences the water activity of the food product. From moisture sorption data collected at different temperatures, the

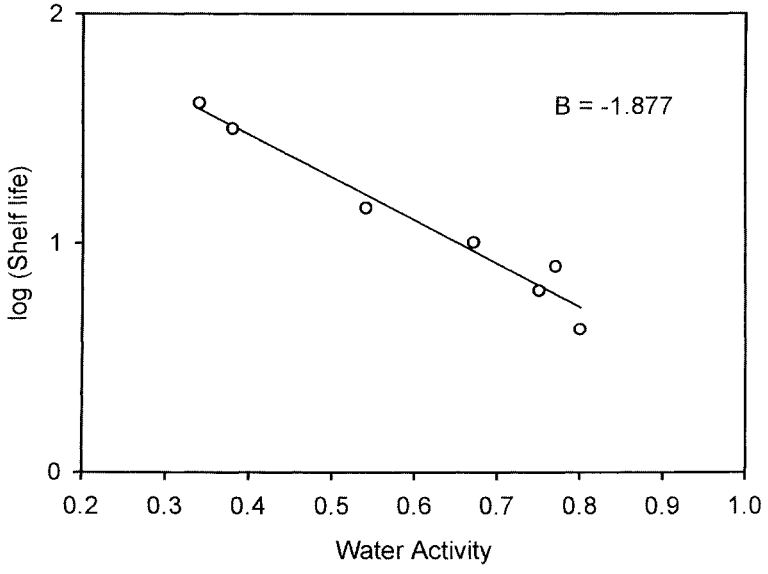

FIGURE 30.8 Q_A plot for aspartame shelf life at pH 5 and 30°C. (Data from References 21 and 40.)

Clausius–Clapeyron equation, shown below, can be used to predict the water activity at a constant moisture content for the new temperature.[38,39]

$$\ln(a_{W2}/a_{W1}) = -(Q_S/R)(1/T_2 - 1/T_1) \tag{30.18}$$

In the above equation, Q_S is the excess heat of sorption and R is the ideal gas constant.[38,39] A plot of the natural log of water activity as a function of reciprocal temperature in Kelvin yields a straight line, from which the water activity at other temperatures can be extrapolated.[38,39] Accurate shelf life predictions require knowledge of the true water activity at the actual product storage temperature, which may be different from the experimental temperature conditions.

Another issue associated with moisture is its ability to plasticize amorphous solids; aspects of this phenomenon are presented in detail by Roos.[41] Briefly, the conversion of a rigid glassy amorphous solid into a softer more viscoelastic rubbery solid is known as the glass transition. This conversion is promoted by either increasing temperature or adsorbing moisture. The glass-to-rubber transition can promote unwanted crystallization of sugars, softening of crispy bakery products, and clumping of powders, making such products unacceptable. In addition to physical changes, the glass transition may impact chemical stability; much research is continuing in this area to elucidate potential effects more clearly. Nevertheless, the glass transition can cause a product to become unacceptable such that the product is not consumed. As mentioned earlier, physiological effects of nutraceuticals are only achieved if the product is consumed.

As discussed, moisture transfer within a product or between the product and the exterior environment can lead to a loss of shelf life. Moisture gain can cause enhanced chemical reactivity as well as undesirable physical changes (stickiness, loss of crispness). Thus, the selection of packaging to reduce moisture gain (or loss) is important for maximizing nutraceutical stability. Understanding moisture sorption isotherms allows for proper package selection and the ultimate control of moisture transfer. The use of anticaking agents, such as calcium silicate, can help reduce undesirable clumping of powders while the use of humectants (e.g., glycerol, sucrose, salt) can reduce water activity.[38] Handling instructions can also help by informing consumers of the importance of properly resealing packages of partially consumed nutraceutical products. Controlling undesirable effects associated with moisture is the second approach toward improving nutraceutical stability.

C. Effect of Oxygen on Stability

Several nutraceutical ingredients are sensitive to oxygen. Polyunsaturated fatty acids, β-carotene, and vitamin C are all subject to enhanced degradation due to oxidation.[42] For example, water containing vitamin C and 5 ppm copper at pH 3.2 and 30°C lost 30% more vitamin C when shaken for 30 min (oxygen incorporated) as compared with unshaken solutions.[43] For lipid oxidation, the effect of oxygen on the oxidation rate, R, has been expressed as

$$1/R = (1/k_1)\{1 + ([O_2]/k_2)\} \tag{30.19}$$

where k_1 and k_2 are rate constants and $[O_2]$ is the oxygen concentration.[28] At low oxygen concentrations, the oxidation rate increases linearly with increasing oxygen concentration. Above a critical oxygen concentration, the rate becomes independent of oxygen concentration. More information on the kinetics of lipid oxidation can be found elsewhere.[28,29]

If oxygen-sensitive substances exist in the nutraceutical, proper package selection and the use of antioxidants can help extend product shelf life. Encapsulating these ingredients to shield them from oxygen may also be beneficial. Oxygen scavengers, packets of iron enclosed in the product package, also keep the oxygen content low, thus helping prevent undesirable oxidation.[44]

D. Effects of Ingredients on Stability

Nutraceuticals and functional foods contain more than just the bioactive ingredient in the formulation. These products often contain amines (e.g., amino acids, proteins), carbonyls (e.g., sugars, flavors), minerals, and buffer salts among other substances. These substances can have dramatic effects on the chemical stability and ultimate acceptability of the nutraceutical.

Amines and carbonyls react via the Maillard reaction to cause flavor modification, amino acid destruction, and brown discoloration. If the bioactive substances in nutraceuticals contain amine or carbonyl groups, the possibility exists for them to degrade via the Maillard reaction. Even without loss of the bioactive substance, other quality changes (e.g., browning, off-flavors) that reduce the likelihood of consuming the nutraceutical result in the consumer not obtaining the expected physiological effect from the product.

The presence of minerals, either intentionally added or naturally occurring, can influence product stability. Lipid oxidation is catalyzed by the presence of minerals (e.g., iron, copper), which results in potent off-flavors and aromas.[28,29] A small amount of such oxidation can result in an unacceptable product. Similarly, vitamin C degradation is also catalyzed by metal ions. For example, as the concentration of copper increased in water, the amount of vitamin C remaining dramatically decreased.[43] Thus, fortifying a nutraceutical with both vitamin C and a mineral, such as iron, can lead to enhanced vitamin C degradation and nutritional labels which are no longer in accordance with federal regulations.

Buffer salts are often added to foods for controlling the pH; however, these can also have significant effects on chemical reactivity. Phosphate buffer enhances the degradation of aspartame as compared with citrate buffer, and the effect increases as the buffer concentration increases.[45] Phosphate buffer was also shown to increase the loss of glycine and extent of browning associated with the Maillard reaction.[46] Thus, the selection of buffer type and concentration can have serious implications for nutraceutical product stability.

III. ACCELERATED SHELF LIFE TESTING

Although it is desirable to investigate chemical stability in a system at conditions as close to those of the commercial product as possible, often the time constraints of product development require the use of accelerated shelf life testing for predicting product stability. Accelerated shelf life testing

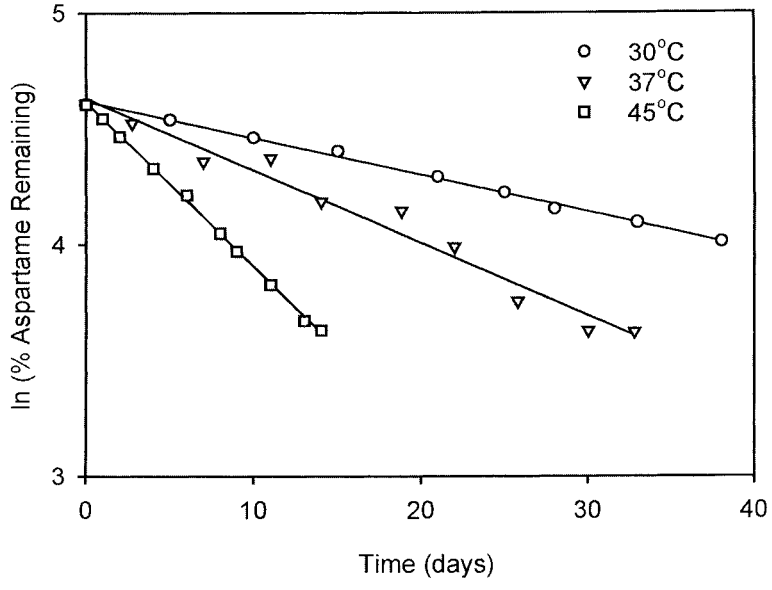

FIGURE 30.9 Pseudo-first-order kinetic plots of aspartame degradation in 0.1 *M* phosphate buffer at pH 3 and various temperatures. (Data from Reference 16.)

utilizes extreme conditions to enhance shelf life loss followed by an extrapolation to normal conditions. The extreme conditions may include high temperatures and/or high water activities. The benefit of this protocol is that answers come faster, saving time and money, but at the risk of some errors.

The best way to understand accelerated shelf life testing and shelf life prediction in general is to discuss two examples. The first example is aspartame degradation in 0.1 *M* phosphate buffer at pH 3, where the data were collected from 30 to 45°C.[16] Figure 30.9 shows the degradation data, modeled using pseudo-first-order kinetics. Nine to ten data points were collected to at least 50% degradation. The slopes of these plots are the pseudo-first-order rate constants. Figure 30.10 shows the Arrhenius plot of the aspartame kinetic data, from which pseudo-first-order rate constants at any other temperature can be mathematically extrapolated. At 25°C, the extrapolated rate constant of 0.00924/day was found to be the same as that determined experimentally (0.0089 ± 0.0011/day).[45] For refrigerated storage at 4°C, the extrapolated rate constant is 0.000802/day. If one assumes an initial aspartame concentration of 600 ppm and that the product remains acceptable to a final concentration of 500 ppm, then the shelf life, t, at 4°C is calculated using pseudo-first-order kinetics as shown below:

$$\ln(500/600) = -(0.000802/\text{day})(t) \tag{30.20}$$

Equation 30.20 yields a shelf life of 7.5 months, which would require too long a time to determine experimentally for most businesses.

The second example uses aspartame degradation data from 0.01 *M* phosphate buffer at pH 7, but collected from 70 to 100°C.[22] Using the Arrhenius relationship (Equation 30.12), the pseudo-first-order rate constant for aspartame degradation at 25°C was calculated to be 0.042/h. The experimentally determined rate constant (0.014/h)[45] is much lower than that predicted using the high temperature data. This result means that the true shelf life is three times longer than that predicted by accelerated shelf life testing. In this case, the product would be assumed to have deteriorated three times sooner than it really would, resulting in acceptable product being discarded. The opposite effect is possible as well, where the shelf life is shorter than expected. These are two potential problems associated with accelerated shelf life testing.

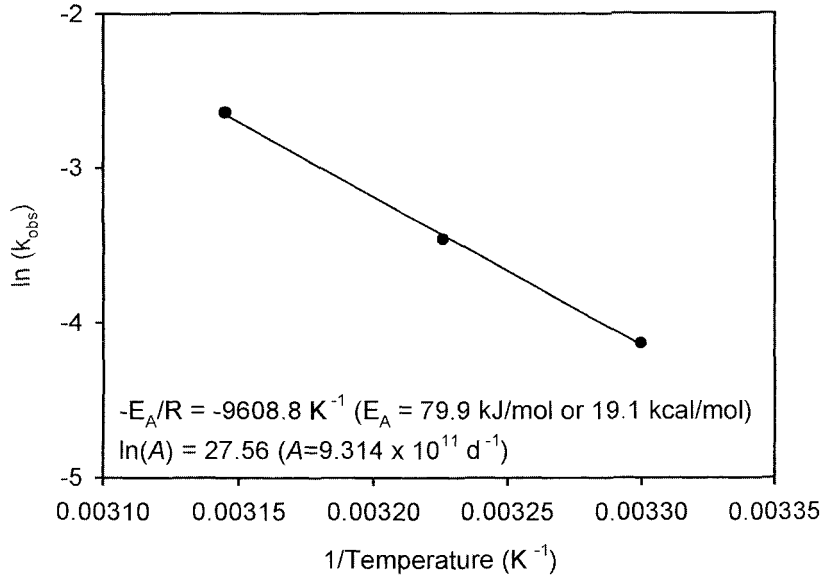

FIGURE 30.10 Arrhenius plot of aspartame degradation in 0.1 *M* phosphate buffer at pH 3. (Data from Reference 16.)

The under- and overprediction of shelf life, as demonstrated previously, can be attributed in part to changes in water activity, pH, and reactant solubility as a function of temperature. In addition, thermal transitions (e.g., glass transitions and lipid melting) can impact predictions from high to low temperatures. Another problem associated with accelerated shelf life testing is competing chemical reactions. The activation energies of various chemical reaction types differ. Thus, two reactions with different activation energies will each predominate over a different temperature range. A critical crossover temperature exists as shown in Figure 30.11. Below this temperature

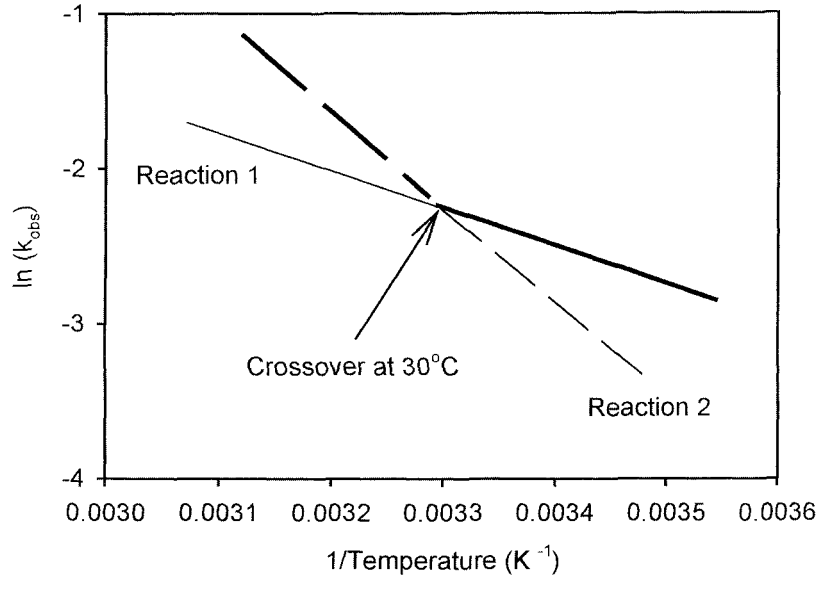

FIGURE 30.11 Arrhenius plot of two reactions occurring simultaneously in a nutraceutical. The bold lines represent the region where each reaction predominates.

(30°C), reaction 1 predominates and is responsible for shelf life loss; however, above this temperature, reaction 2 predominates. For example, oxidation of polyunsaturated fatty acids may be the major mode of product deterioration at one temperature, whereas vitamin degradation predominates at another temperature. The possibility of multiple modes of nutraceutical deterioration needs to be recognized before using accelerated shelf life testing. The critical reaction responsible for loss of shelf life under typical storage conditions should be identified and utilized during the shelf life test. Although accelerated shelf life testing has its advantages, the potential errors need to be recognized. Other aspects of accelerated shelf life testing are discussed by Labuza and Schmidl.[47]

IV. FINAL THOUGHTS

Shelf life testing is an important aspect of nutraceutical product development. The consumer demands a quality product, and that is possible only by optimizing the product shelf life. If the biological activity or sensory attributes deteriorate, the product has failed to meet consumer expectations. Other implications associated with stability issues include failure of the product to meet label claims, which is a violation of federal law. Optimizing stability requires, at the very least, the ability to control temperature abuse and moisture transfer. Solutions for each of these have been presented. By using a well-designed experimental plan and appropriate kinetic modeling, accurate shelf life predictions can be made, benefiting both the company and the consumer.

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31 Marketing Issues for Functional Foods and Nutraceuticals

*Nancy M. Childs**

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* Portions of this chapter were adapted and updated from the author's chapter "Marketing Functional Foods: What Have We Learned? An Examination of the Benefit and Heartwise Introductions as Cholesterol Reducing RTE Cereals"[1] and from the author's chapter "The Functional Food Consumer: Who Is She and What Does She Want? Implications for Product Development and Positioning," in *New Technologies for Healthy Foods and Nutraceuticals*.[2]

0-8493-8734-5/01/$0.00+$.50
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I. INTRODUCTION

During the 1990s functional foods and nutraceuticals emerged as the dominant trend for the food industry, both in the United States and internationally. The concept of foods that could provide health-enhancing and disease-preventing properties was embraced by a growing number of consumers, increasingly documented by nutritionists and scientists, and legally endorsed by public policy and legislative mandates for food and dietary supplement labeling. These developments spawned considerable corporate attention across several industries, from agricultural and biotechnology life science concerns that grow and develop the raw commodities to nutritional, food, and pharmaceutical manufacturers that design new products.[3] Bringing these newly developed and newly positioned products to the consumer was the challenge and the value-added opportunity pursued by these industries.

While consumer interest in the category continued to grow, 1999 emerged as a year with strong market gains for foods using Nutrition Labeling and Education Act (NLEA) approved health claims and nutrient content claims as part of their marketing message. Product successes of note included the volume gains reported by Quaker Oats in its third quarter financial report featuring 7% gains for oatmeal for the summer quarter, a traditional down period for hot cereal consumption. Ready-to-eat cereals increased 5% in volume for the period.[4] General Mills, in its mid-year report, highlighted 13% volume gains for "Cheerios" using the oat bran and then the whole-grain health claim. Other General Mills whole-grain cereals received similar double-digit volume gains in a food category dependent on population increase (0.6%) for category growth.[5] Campbell Soup Company reported impressive success with their "V8 Splash" line, rich in antioxidants, which exceeded expected market growth and surpassed its category parent "V8" vegetable juice.[6]

Marketing of products with health claims and reaching consumers with meaningful messages and positionings for products in the functional food arena take careful attention to regulatory issues and insightful thought on how to communicate product benefits for consumer attention. Regulations control the language and scientific benefit that may be conveyed on the product label, in the marketing literature, and in the product advertising. Consumers, however, are often more persuaded by nuance, association, and promises of well-being than by scientific statements and numerical documentation.

II. EVOLUTION OF A MARKETING ENVIRONMENT FOR FUNCTIONAL FOODS AND NUTRACEUTICALS

Since the 1970s food has taken on major connotations of being "good-for-you" and "bad-for-you" foods, the latter, such as saturated fats and sodium, to be avoided or ingested in moderation. The "good-for-you" foods increasingly include foods and food components shown to lower the risk of cancer, heart disease, and other chronic diseases of aging. Since then, numerous studies and research reports have been published documenting the association between diet and health. In response, public health goals reoriented from prevention of diseases associated with nutritional deficiencies to an emphasis on nutrition for decreasing risks for chronic disease (Table 31.1). Early attention focused on preventing coronary heart disease, stroke, high blood pressure, cancer, diabetes, obesity, osteoporosis, dental diseases, and diverticulum disease, among others, as pursued in the Surgeon General's landmark document on nutrition and health published in 1988.[7]

During the Bush administration two major initiatives dominated consumer nutrition policy. The Food and Drug Adminstration (FDA), acting under Congress's directive, wrote new regulations governing health claims on food labels. The NLEA of 1990 became effective in 1993. With the assumption that the food label is a primary nutrition education vehicle for the consumer, the NLEA carefully restricted what can be claimed on the label as well as what nutritional information must be disclosed.

TABLE 31.1
Government Nutritional Policy Promoting Consumer Education

1988 – Surgeon General's Report on Nutrition and Health
1989 – Academy of Science Nutrition Guideline
1990 – Nutrition Labeling and Education Act (NLEA) enacted
1991 – Institute of Medicine Food Guidelines
1992 – USDA Food Guide Pyramid
1993 – NLEA regulations issued by FDA
1994 – RDA reexamination proposed
1994 – Dietary Supplement Health and Education Act (DSHEA) enacted
1996 – Dietary Guidelines for Americans
1997 – Calcium RDI released
1997 – FDA Modernization Act enacted
1997 – DSHEA commission report issued

The second parallel consumer nutrition education thrust involved the redefinition and reissue of the U.S. Department of Agriculture (USDA) Five Food Groups from 1979 as the initially controversial Food Guide Pyramid released in 1992. The new Food Guide Pyramid recommends the number of servings for six food groups.

In this environment emphasizing the role of diet in preventing disease and in promoting good health, a marketing, consumer, and regulatory crisis began to surface. Consumer interest and education in food components that prevent disease and prolong good health, accompanied with publicized technological advances and scientific studies isolating food components such as antioxidants and carotenoids whose presence in food delivers these prophylactic benefits, have created a market for such products. These products have been referred to by many titles including nutraceuticals, functional foods, designer foods, and other labels from the corporate and scientific community. Interested consumers seek and respond to marketing claims that identify and elaborate on these components. Such claims, particularly as presented on the product label, created a growing dilemma to the FDA in the mid- and late-1980s.[8–10]

III. REGULATORY BACKGROUND

Since 1973, FDA regulations have stated that a food whose labeling represents that the food is adequate or effective in the "prevention, cure, mitigation, or treatment of any disease or symptom" is deemed "misbranded." These regulations were amended in 1993 to exempt FDA-approved health claims (21 C.F.R. 101.9(i)(1)(1992 ed.; recodified and extended in new NLEA regulations at 21 C.F.R. 101.9(k)(1), 58 *Fed. Regis.* 2533, January 23,1993).

A. Appearance of Permissive Health Claims on Food Products

Contrary to the strict pre-1993 provisions, in the mid-1980s the FDA pursued a policy of selective nonenforcement, permitting an acceleration of explicit health-related and disease-related claims on food products that the FDA felt were justified and benefited the public health. The frequently cited "watershed" was the 1984 promotion of "All Bran" cereal by Kellogg's Company with labels that explicitly claimed preventive benefits of fiber with respect to cancer: "... eating the right foods may reduce your risk of some kinds of cancer ... eat high fiber foods ... bran cereals are one of the best sources of fiber."[11–13]

This promotion was jointly conducted with the National Cancer Institute (NCI) which in the 1980s and 1990s was ahead of other government agencies in promoting to the consumer the use

of diet, and specifically foods rich in certain nutrient properties. The NCI launched the well publicized Designer Foods Program specifically to address and document the role of these phytochemicals in cancer prevention.[14]

Other permissive claims in the late 1980s included oat bran claims concerning lowered blood serum cholesterol and the reduced risk of chronic heart disease for oat-based breakfast cereals and other products containing oat bran; claims that calcium helps reduce the risk of osteoporosis promoted on dairy products and dietary supplements; and vegetable oil products posting a variety of claims from "cholesterol-free" to "better for your heart than" to specific claims regarding lower blood serum cholesterol and reduced risk of chronic heart disease. By 1990, it was reported that "... 40 percent of all new food products introduced in the first half of 1989 bore general and specific health claims."[15]

Besides the exploding number and variety of health claims promoted by food marketers, which the FDA choose to ignore, thereby informally condoning them, the FDA also officially exempted several food categories from drug status and accountability while permitting explicit health- and disease-related labeling claims. These food product categories included "medical foods," "hypoallergenic" foods, diabetic foods, sugarless foods that "will not promote tooth decay," and foods that are qualified for special dietary uses (21 C.F.R.).[16]

B. Reaction and Institution of the NLEA

The incipient loss of control by the FDA with its official and unofficial exemptions encouraged a flood of health claims from entrepreneurial marketers and created a consumer marketplace rife with confusion and skepticism. Adding to the loss of control and embarrassment on the FDA level was the ambitious behavior of several state attorneys general who very publicly invoked their authority in this arena, on behalf of consumer protection, claiming fraudulent and misleading food labeling and seizing product.[17–19]

An early attempt to rope in this imitative, ambitious marketing behavior was an FDA proposal in 1987. This set of permissive guidelines never had effect and ultimately was replaced by the restrictive 1990 NLEA proposals. The strict separation of definition of food and drug returned. A food product could make a health- or disease-related claim "only if (FDA) determines, based on the totality of publicly available scientific evidence (including the evidence from well designed studies...) that there is significant scientific agreement, among experts ... that the claim is supported by such evidence" (21 U.S.C. 343(r)(3)(B)).

While the overall regulations were quite stringent, more restrictive, more inclusive, and set high standards for qualification, the FDA did address and rule on ten claims that the agency examined for authorization. The authorized claims were approved for use as generic health claims on foods that qualified. Table 31.2 lists the originally approved health claims under the NLEA. The FDA also established a definition for the term *healthy* for use in food labeling. To be labeled as healthy, food must meet the definition of "low" for fat and saturated fat, must contain cholesterol and sodium below disclosure levels prescribed in FDA regulations, and must comply with all applicable rules concerning specific nutrient content claims on the label (21 C.F.R. 101.65(d)(2), 58 *Fed. Regis.* 2944).

This select and limited approach permitted access to generic health claims in the food situations that qualify under the authorized categories listed in Table 31.2. Any health claim that was not explicitly approved for use in food labeling by an FDA regulation was deemed to be forbidden from use in food labeling.[20]

The implementation of the NLEA in 1993 quickly prompted concerns of ambitious enforcement from the FDA. In hindsight, two results are easily documented. Industry's adherence to the new legislation dramatically reduced the number of claims used in food product advertising (Figure 31.1) and the concerns of the dietary supplement industry that the FDA would aggressively apply the new regulations to their growing industry prompted separate legislation. The quick and unanticipated

TABLE 31.2
Health Claims Receiving Approval under NLEA

Seven Health Claims Receiving Original Approval under the NLEA

- Calcium and osteoporosis
- Sodium and hypertension
- Dietary fat and cancer
- Dietary saturated fat and cholesterol and risk of coronary heart disease
- Fiber-containing grain products, fruits, and vegetables and cancer
- Fruits, vegetables, and grain products that contain fiber, particularly soluble fiber, and risk of coronary heart disease
- Fruits and vegetables and cancer

Additional Health Claims Receiving FDA Approval under the NLEA

- Folate and neural tube birth defects
- Dietary sugar alcohol and dental caries
- Dietary soluble fiber, such as that found in whole oats and psyllium seed husk, and coronary heart disease

Source: Adapted from *J. Med. Foods*, 1(3):236, 1998.

passage of the Dietary Supplement Health and Education Act (DSHEA) in 1994 created a separate set of label criteria for the ancillary dietary supplement industry that was distinct from the regulations required on food labels. It also created a separate and distinct regulatory category for dietary supplements with separate marketing standards for label claims and advertising statements.

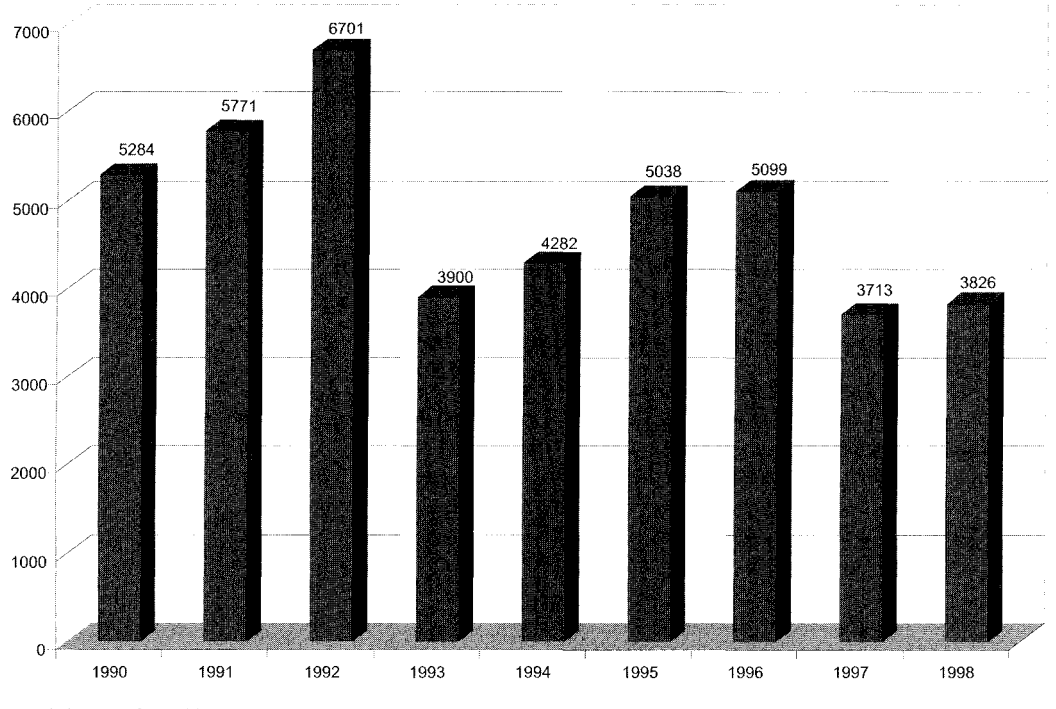

FIGURE 31.1 Use of nutrition messages on food labels; nutrient/content–structure/function–health claim. (Adapted from *New Products News*.)

The regulated market for health claims on foods and claims on dietary supplements continued to evolve in the late 1990s as claims were approved and challenged, and as various legal precedents occurred in the environment. The Food and Drug Modernization Act (FDAMA) of 1997 permitted the consideration of authoritative statements as a source for health claims on foods and led to the approval of the whole-grain health claim in 1999. Legal developments during 1999, many still under review, expand the possibility that manufacturers may be able to bring more "emerging" science to the label as they present their data in unbiased terms.

One of the two key distinctions that remain in the regulatory environment is pursuit of claim approval, which includes the amount of documentation and time required for approval. The second is the type of claim and the structure of the communications message permitted. For a health claim to be approved on a food product, the claim must be submitted to the FDA with full scientific documentation. All materials enter the public domain. After lengthy review and public comment, estimated to be in excess of 500 days, the claim may receive generic approval for use by all products that meet the qualification of the claim. A health claim permits a statement linking the nutrient with a disease state. A dietary supplement claim is limited to a structure–function statement connecting the nutrient to good health. It can be used on the label and requires a 90-day postmarketing submission to the FDA. Substantiation is necessary for the claim, which also is generically available for qualifying products. There are substantial and critical marketing differences in the two claim approval formats and the language structure permitted by the two regulatory structures for foods and dietary supplements.

IV. INTRODUCTION TO CONSUMER MARKETING ISSUES FOR NUTRACEUTICALS AND FUNCTIONAL FOODS

Nutraceutical and functional food consumers tend to be female, middle-aged, affluent, and more educated than the average consumer. They represent a desirable marketing segment that is less price sensitive and more proactive regarding pursuit of good health. They segment into numerous lifestyle and behavior groups, and have concerns across a number of chronic disease states.[10,11] They represent a quintessential target market.

Marketing issues can be divided between general factors promoting marketing success, as listed in Table 31.3, and specific factors for product positioning on a whole health continuum. General factors, or lessons learned for marketing foods utilizing health claims, are discussed below.[10]

TABLE 31.3
Lessons Learned for Marketing Functional Foods

- Good taste necessary
- Brand name should connect to functional advantage without compromising taste assurance
- Consumer education required
- Consumer confused by information overload and contradiction in media
- Avoid complicated claims referencing numbers or unfamiliar food components
- Competitive set determined by health issue, not product category
- Nonverbal messages are important: satisfied users
- Suggest usage occasion and product substitution
- Avoid negative or scare-tactic advertising
- Functional foods serve niche markets
- Recognize and exploit corporate heritage in specific product categories
- Provide assurances for dosage and standardized product

A. Good Taste Necessary

Most successful new food products in the late 1990s demonstrated three criteria: taste, convenience, and nutritional advantage. Taste remains paramount of the three and the dictating factor for repeat purchase. Although nutrition or convenience may generate trial purchase, neither will sustain repeat purchase without good taste. Not only should nutraceutical products provide good taste, they must promise good taste in their advertising and reinforce the consumer's curiosity for good taste.

B. Brand Name Connects to Functional Advantage

The brand name of the product must connect to the health benefit of the product and offer insight into the unique functional value of the food, as well as not connote a taste concern. An exception would be a preexisting product like oatmeal repositioned for its functional health value, where the preexisting brand equity is retained. The early psyllium cereals demonstrated the communication strength of the more straightforward "Heartwise" brand offered by Kellogg's vs. the more vague implications of General Mill's "Benefit" brand moniker. Again, the Nabisco Brand "NutraJoint" product communicated with consumers while the recent Kellogg's psyllium line "Ensemble" did not equate the beneficial purpose of the product.

C. Consumer Education Required

The more specific the nutrient and its benefit, the more consumer education on the disease state and health condition is needed. While cardiovascular disease, cancer, and osteoporosis seem to be reasonably well communicated health concerns, many of the more specific health issues are not. Complementarily, the nutrients have varying levels of understanding by consumers. Popular antioxidant vitamins are readily accepted for their health value, whereas newer antioxidants such as lycopene and xianthan are less familiar and not readily identified with the health condition they sustain. Levels of consumer education appear to follow public health campaigns, and to the degree that approved health claims receive rapid exposure in the commercial and public health media, consumers quickly appreciate new nutrient benefits.

D. Avoid Information Overload

Consumers do not easily process complicated quantitative messages regarding nutritional benefit, and do not readily understand comparative mathematical relationships as validation of nutrient benefit. Although such information may be provided on the label and in the marketing literature for the product, it should not be the essence of the positioning and advertising of the product. Such information is useful for the medical audience and the informed consumer and its presence provides value for these purposes.

E. Competitive Set Determined by Health Issue

One of the most repeated fundamental marketing errors that has occurred with functional foods and nutraceuticals is the inclination to address the product category competitive set and not the substitutive competitive set defined by the health condition. Cholesterol-lowering foods, whether oatmeal, soy, or stanol ester spreads, are in direct competition with ethical drug products for this purpose and provoke strong competitive response from the pharmaceutical industry. These defensive responses are addressed to both the consumer and the medical community. Food manufacturers rarely anticipate this out-of-category, indeed, out-of-industry, counterresponse.

F. Non-Verbal Messages Important

The use of nonverbal messages is an important method for communicating good taste, quality assurance and functionality. Product functionality can be connoted in a number ways by showing active, satisfied users. Taste assurance is conveyed with satisfied users enjoying the product. Nonverbal messages also can be used to suggest target market consumers, product quality, and to provide a "natural" halo for the product with visuals of fields, growing plants, plant botanical graphics, or other reassuring images.

G. Usage Occasion

The sight of satisfied users and the presence of the product consumed in a usage occasion environment is reassuring and provides context to the consumer. Usage occasion is an important factor as it suggests to the consumer the method for incorporating the product into a daily routine, thereby meeting dosage demands. It also suggests product substitution possibilities, which increase likelihood of product adoption.

H. Avoid Negative Advertising

To date, advertising focusing on the fear of the disease and the loss of health has not resonated with consumers. Consumers are wary of avoidance messages, and are more favorable and receptive to messages of "more" and "better" and general promises of good health. Functional foods are excellent sources of "better" and "good-for-you" product messages and are easily positioned to emphasize their positive advantage. This good health approach also is less challenging to the regulatory structure.

I. Niche Markets

Almost by definition, as bioactive ingredients are better understood, they are promoted for specific use by specific populations and represent niche market opportunities. As the category matures, competition increases, and the medical community becomes more interested in products, the niche market specificity will become more important. These specifics are necessary for product differentiation, superiority, and credibility.

J. Exploit Corporate Heritage

Many food and pharmaceutical companies hold enviable brand equity positions, such as "Quaker Oats Oatmeal" mentioned earlier, and enjoy corporate images of trust and expertise with the consumer. McNeil Consumer Health holds such a trust with the consumer as does Kellogg's with its healthy fiber cereal dominance. Kellogg's Heartwise cereal was able to secure ready consumer acceptance as a high-fiber cereal because of the Kellogg's heritage in the category. For General Mills' Cheerios brand and Tropicana's "Pure Premium Plus" line, the existing equity of taste and quality were powerful foundations for their transformations to functional food positionings. The existence of a product line or portfolio allows the nutrition message and health claim to be directed at one product in the line while the health "halo" covers the entire line, even the items that are sugar frosted, marketed to children, or otherwise inappropriately positioned to be consonant with targeted health claim marketing.

K. Dosage and Standardization

As the category for functional foods continues to mature, the regulatory environment, the educated consumer, and the medical community will be asking for levels of bioactive presence to fulfill efficacious dosage levels. Standardized product will be an important factor for assessing product quality, and perhaps even to meet required product certification in the future.

V. POTENTIAL PRODUCT POSITIONING

Consumers interested in functional foods and nutraceuticals have four categories of product function that are desired. These are therapy, prevention, performance, and particularly in the United States, weight loss. The therapy, prevention, and performance categories have varying foci depending on the respondent's sex and age.

Yankelovich data[21] identifies 40 million consumers who are "health active," meaning they act today to ensure good health when older, are concerned about family nutrition, regularly eat fruit, accept medications, and exercise twice a week. The study also recognizes a somewhat similar group of consumers, titled "health aware" who are like the former "active" group except they do not exercise twice a week. This group, also comprising consumers over age 18, is estimated to include 13 million consumers. These are sizable consumer markets.

Of notable interest is that these "health active" and "health aware" consumers also differ from the remaining "health uninvolved" population of 137 million people by virture of their advanced interest in the continuum of Food–Nutrition–Health. They are far more likely to have moved farther along the continuum to Food–Nutrition–Health–Wellness-Well-Being. Their concept of health has a totality about it that is likely to encompass community, self-enhancement, religion, personalization, rituals, and the environment. These factors can be addressed as physical, emotional, spiritual, social, and financial dimensions.[22] Not surprisingly, people who are willing to believe what they do and eat today may impact their health potentially far in the future have some distinguishing beliefs that can become the foundations for functional food positionings, advertising messages, promotional opportunities, and product ingredients.

A. Physical Components

Functional food and nutraceutical positionings, regardless of product function or product form, exist along each of the five identified dimensions. The physical dimension is the most obvious and is clustered around an attention to nutrition, exercise, and medicine. Functional foods can be positioned along a continuum of nutrition to medicine depending on their scientific credibility and purpose for therapy, prevention, or performance. Any products that complement or enhance the healthy benefits of physical exercise offer strong positioning opportunity.

B. Emotional Components

The emotional component involves the needs for nurturing, self-knowledge, and stress management. Functional food and nutraceutical positioning is compatible with a need for nurturing one's own health as well as that of one's family. The woman's proclivity for functional food products encourages her, as primary shopper, to respond to a positioning that nurtures and protects her family. This consumer's interest in self-knowledge, which will include genome vulnerabilities and environmental exposure, as well as the individual's need for stress management, will heighten consumer interest in products, particularly customized products, that address these concerns. Self-knowledge and customized/personalized/individualized products offer a potentially powerful match. Test kits and other measurements and "surrogate markers" (such as cholesterol for cardiovascular disease) become valuable vehicles for recognizing individual needs for protection, restoration or enhancement of performance, and recovery. These vehicles will document the need for a specific disease-associated functional food, and thereby encourage its use.

C. Well-Being Components

The consumer's dimension of well-being, or spirituality, encompasses meditation, prayer, energy, and nature. The two latter components directly relate to the performance purpose of functional foods (energy) and to the desirability of natural ingredients for such products (nature). The term

natural takes on many dimensions of purity from the desirable organic stricture to being naturally sourced, or simply being plant derived rather than a laboratory-sourced synthetic substitute. Consumers' first preference, by a wide margin, is to obtain nutraceutical substances through consumption of fruits and vegetables. This underscores their desire for familiar and natural sources for these active ingredients. Natural connotations can be conjured from the product name, label graphics, and advertising setting. In Japan, where the culture harmonizes rather than separates food and health, the FOSHU (Foods for Specified Health Use, category which replaced the former functional food category) products are required to be of natural origin.

D. Social Components

The social component includes three factors of large and familiar promise for functional food positioning. These are family, community, and philanthropy. The first two provide reasons as well as occasions for functional food consumption. So long as nutraceuticals remain a functional food, an edible or drinkable product, they have the potential to be a part of the most social and most routine parts of our lives, namely, daily sustenance. The philanthropy connection is an important insight for marketers as it indicates that relationship marketing should be a powerful tool. Tying products in with preexisting disease foundations and fund-raisers, such as the breast cancer annual "Run for the Cure" event, should carry strong credibility to these consumers. Foundation endorsements, seals of approval, and spokespeople offer potential marketing and positioning tactics. Many may offer opportunities for exclusivity, which could become a powerful product point of differentiation in an arena of generic claims.

E. Financial Components

The fifth dimension is financial and it clusters into four drivers: concerns and preparations for comfort, for retirement, for maintaining independence from one's children, and for contingency planning. While obviously no functional food is a financial investment instrument, brand positionings, nonverbal advertising messages, and promotions that focus on the enjoyment and attainment of these goals give a credible context and purpose for preventive functional food products. This is already evident in the advertising campaigns for Quaker Oats Oatmeal using the health claim language in a venue of active seniors enjoying breakfast on the golf course; Tropicana Pure Premium Plus presenting active seniors hiking under the headline, "Leave the Grandkids in the Dust" and inclusion of the health claim; and in "Ensure's" active senior advertising. Ensure's campaigns have focused on two of the above elements, elder parent–grown child enjoying the product together and toasting "to our health" and implicit independence and, more recently, a campaign of active seniors, again emphasizing the comfort and fun opportunities offered by healthy retirement.

VI. SUMMARY

In summary, product positioning potential is multidimensional for a segment of consumers of proclaimed belief in functional food and nutraceutical products. This target market responds to product purpose for prevention, therapy, and performance. They have specific disease states that concern them, in an individualized manner, for which they are receptive to seeking prevention and therapy products. Such disease concerns include heart disease, cancers, Alzheimer's, and others. As well as the segments of product purpose and target disease, described above, there are product positionings and advertising messages, verbal and nonverbal, that carry particular credibility and immediacy to these consumers. These tend to cluster into the five areas elaborated above which include physical, emotional, spiritual, social, and financial components. Each offers numerous product "hooks" for catching consumer interest with a credible and identifiable message. While not offering specific direction for product function, form, or targeted disease state, these components

offer marketing context for product positionings, insight for product names, label design, advertising campaigns, promotions, comarketing, spokespeople, endorsing organizations, and other marketing tactics. Even though regulation does not permit proprietary health claims, powerful marketing relationships can be structured in an exclusive manner. This is a familiar aspect of sports drink marketing, a performance product category.

VII. CONCLUSION

Nutraceuticals and functional foods are clearly poised as a 21st-century industry. They promise value-added opportunities in the food industry and new market opportunities for the pharmaceutical industry. They offer advances in public health as health claim marketing messages empower consumers to select healthier food choices. Regulatory issues are complex and have evolved in a politically driven fashion with three major legislative efforts transpiring in the 1990s (NLEA, DSHEA, and FDAMA) and several legal decisions impacting regulatory interpretation and application.

The regulatory activity defines the marketing parameters for the product label which is one of the marketing venues. Several suggestions are given for savvy marketing of functional food products. Elaboration on product positionings are offered acknowledging that consumer receptivity often hinges on perceptions of quality, taste, acceptability, and well-being rather than specific product potency and benefit.

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