METHODS OF BIOCHEMICAL ANALYSIS

Volume 36

METHODS OF BIOCHEMICAL ANALYSIS

Series Editor

Clarence H. Suelter

Volume 36

BIOANALYTICAL APPLICATIONS OF ENZYMES

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SERIES PREFACE

Methods of Biochemical Analysis was established in 1954 with the publication of Volume 1 and has continued to the present, with volumes appearing on a more or less yearly basis. Each volume deals with biochemical methods and techniques used in different areas of science. Professor David Glick, the series' originator and editor for the first 33 volumes, sensed the need for a book series that focused on methods and instrumentation. Already in 1954, he noted that it was becoming increasingly difficult to keep abreast of the development of new techniques and the improvement of well-established methods. This difficulty often constituted the limiting factor for the growth of experimental sciences. Professor Glick's foresight marked the creation of a unique set of methods volumes which have set the standard for many other reviews.

With Professor Glick's retirement from the series and beginning with Volume 34, I have assumed editorship. Because the rationale used in 1954 for the establishment of the series is even more cogent today, I hope to maintain the excellent traditions developed earlier. The format of Volume 34 and later volumes, however, is changed. Rather than cover a variety of topics as previous volumes did, each volume will now focus on a specific method or the application of a variety of methods to solve a specific biological or biomedical problem.

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PREFACE

Volume 36 of *Methods of Biochemical Analysis* focuses on the bioanalytical applications of enzymes. Because enzymes facilitate rapid and highly specific molecular transformations under mild conditions, this class of protein has become increasingly important in analysis, synthesis, manufacturing, and medical diagnosis.

The introductory chapter of this volume describes selected applications of enzymes in analytical chemistry and presents an update on enzyme immobilization procedures. Immobilized enzymes are used increasingly for synthesis, degradation, and analysis of many analytes. They provide great flexibility, particularly in the design of enzyme-based bioanalytical systems. Chapter 2 describes the use of immobilized enzymes in dry reagent chemistry elements. This chapter portrays the multiplicity of scientific disciplines involved in element construction, the use of reflectance photometry in monitoring assays. The history, fundamental principles, fabrication, performance and application of enzyme electrodes (biosensor technology) are described in Chapter 3. Chapter 4 presents a review of the properties of enzymes used in preparing DNA and RNA probes for hybridization. Reaction conditions typically employed, and examples of types of experiments generally performed with different probes are also included. Restriction fragment length polymorphisms (RFLPs) produced by restriction enzymes provide a powerful tool for studying a number of biomedical problems in genetics, neoplasia, and infectious diseases. Restriction enzymes and the use of RFLPs in medicine are discussed in Chapter 5. Chapter 6 presents a review of enzymatically coupled field effect transistors (FETs). The authors include a review of enzyme-immobilized membrane deposition methods, the performance of some FET biosensors, and some recent applications of enzymatically coupled FETs. Chapter 7 discusses the methods available for labeling antibodies with enzymes, some applications of enzyme-labeled antibodies, and their relative merits in analytical chemistry. The amplification properties of an enzyme make it an ideal label in situations requiring extreme analytical sensitivity. Consequently, enzyme labels have been used extensively in bioanalytical methods.

This single-volume compilation of selected bioanalytical applications of enzymes is an ideal reference text for a course in analytical biochemistry.

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ABBREVIATIONS

α-kG	Alpha ketoglutarate
AchE	Acetylcholine esterase
ADH	Alcohol dehydrogenase
AFP	α-Fetoprotein
Ala	Alanine
ALT	Alanine aminotransferase
AOD	Ascorbate oxidase
AP	Alkaline phosphatase
APAAP	Alkaline phosphatase-antialkaline phosphatase
ARIS	Apoenzyme reactivation assay
ASPM	Aspartame
ATP	Adenosine triphosphate
BASC	2,5-Bis(4′-azido-2′-sulfobenzal)cyclopentanone
BSA	Bovine serum albumin
BSNHS	Bis-succinic acid *N*-hydroxysuccinimic ester
CEDIA	Cloned enzyme donor immunoassay
cAMP	cyclic Adenosine monophosphate
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance receptor
cGMP	cyclic Guanosine monophosphate
COD	Cholesterol oxidase
CON A	Concanavalin A
CV	Coefficient of variation
DEAE	Diethylaminoethyl
DNP	Dinitrophenol
DTT	Dithiothreitol
ECFET	Enzymatically coupled field effect transistor
EDTA	Ethylene diaamine tetraacetic acid
EIA	Enzyme immunoassay
EIM	Enzyme-immobilized membrane
ELISA	Enzyme-linked immunosorbent assay

ENFET	Enzyme-immobilized field effect transistor
FAD	Flavin adenine dinucleotide
FET	Field effect transistor
FIA	Flow injection analysis
FMN	Flavin mononucleotide
GA	Glutaraldehyde
γGT	gamma Glutamyltransferase
GDH	Glucose dehydrogenase
GlDC	Glutamate decarboxylase
GlDH	Glutamate dehydrogenase
GlOD	Glutamate oxidase
GMP	Guanosine monophosphate
GOD	Glucose oxidase
GPT	Glutamate pyruvate transaminase
HBsAg	Hepatitis-B surface antigen
HCG	Human chorionic gonadotropin
HEPES	*N*-[2-Hydroxyethyl]piperazine *N'*-[2-ethane sulfonic acid]
HRP	Horseradish peroxidase
Hx	Hypoxanthine
IMP	Inosine monophosphate
ISE	Ion selective electrode
ISFET	Ion-sensitive field effect transistor
kDA	kila Dalton
Km	Michaelis–Menton constant
LAAO	L-Amino oxidase
LDH	Lactate dehydrogenase
LMOD	Lactate mono-oxidase
LOD	Lactate oxidase
LOH	Loss of heterozygosity
NAD	Nicotine adenine dinucleotide
NADP	Nicotine adenine dinucleotide phosphate
NMP^+	*N*-Methylphenazinium
NP	Nucleoside phosphorylase
OAC	Oxalacetate decarboxylase
ODH	Oligosaccharide dehydrogenase
OPDM	B,*N'-o*-phenylenedimaleimide
PAP	Peroxidase-antiperoxidase
PBQ	*p*-Benzoquinone
PBS	Phosphate buffered saline

PCR	Polymerase chain reaction
PGK	Phosphoglycerate kinase
PIPES	Piperazine-*N,N'*-bis[2-ethane sulfonic acid]
PLP	Pyridoxal-5'-phosphate
POD	Peroxidase
POP	Pyruvate oxidase
PVP	Polyvinylpyrrolidone
RFLP	Restriction fragment-lengths polymorphism
RIA	Radioimmunoassay
SCE	Saturated calomel electrode
SOS	Silicon on saphire
TCNQ	Tetracyanoquinodimethane
TdT	Terminal deoxynucleotidyl transferase
Tris	Trishydroxymethylaminomethane
TTF	Tetrahiafulvalene
VNTR	Variable number tandem repeats
XOD	Xanthine oxidase

METHODS OF BIOCHEMICAL ANALYSIS

Volume 36

Unique Applications of Immobilized Proteins in Bioanalytical Systems

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1. INTRODUCTION

Enzymes are being increasingly used in an immobilized form for synthesis, degradation (1), and analysis (2, 3) of molecules. The main advantages of immobilized enzymes (4–6) are reusability and enhanced stability. Although immobilization leads to a decrease in activity and diffusion constraints (4–6), the advantage of reusability outweighs these disadvantages; hence, the applications of enzymes in immobilized form continue to increase. Efforts are also directed toward developing newer methods of immobilization and designing matrices that minimize the adverse effects produced by immobilization.

Immobilization provides great flexibility, particularly in the design of enzyme-based bioanalytical systems. Some relatively recent developments in protein immobilization methodology which can be broadly classified as reversible immobilization methods, have resulted in novel analytical approaches such as bioaffinity sensors (7) and flow injection binding reactions (8).

In this chapter we describe the applications of biological macromolecules in analytical chemistry and present an update on enzyme immobilization procedures (see Everse et al. (9)).

2. REQUIREMENTS OF AN IDEAL BIOANALYTICAL SYSTEM

An ideal bioanalytical system may be characterized in terms of specificity, selectivity, stability, convenience, and cost. Traditionally, most bioanalytical systems have been based on covalently immobilized biomolecules. Some limitations of immobilized reagents have been encountered, and in the efforts to eliminate these constraints alternative procedures have been developed. These developments will be reviewed and compared with traditional covalent immobilization techniques.

Immobilization offers certain advantages concerning stability and reusability. Particle-bound enzymes in flowing systems may have advantages with respect to separation, because many of the technical limitations to designing an

analytical system lie in sample preparation and product separation. Under normal conditions an immobilized preparation is sufficient. But when the medium contains particulate matter or is viscous, then new challenges appear. Some new systems are discussed here, including reversible immobilization, magnetic separation, and extraction in aqueous two-phase systems.

The discussion is split into two main sections: enzyme-based analysis and binding assays.

3. RANGE OF ASSAYS

Any enzyme-based analysis consists of measurement of enzyme activity with or without the presence of substances other than substrate. If these substances, when present, modulate the enzyme activity, that is, if they activate or inhibit the activity, then their amounts can be quantified by using classical Michaelis-Menten kinetics. Thus, an enzyme can be used to assay the following broad classes of substances: substrates, activators, and inhibitors.

4. ADVANTAGES OF USING ENZYMES IN ANALYSIS OF SUBSTRATES

1. Enzymes with reasonably high substrate specificity are available, so it is possible to analyze the desired substance in the presence of many other substances. A tedious specimen preparation procedure is usually not required. Chemical analysis would frequently require such a step, particularly in complex clinical and industrial samples.
2. The conditions of enzymatic methods are milder and thus do not compromise sample stability.
3. Enzymes as reagents are environmentally acceptable.
4. Enzymes can be immobilized, which renders them reusable. Also, the immobilized enzyme reagent can be adapted to various physical forms.
5. The cost of using immobilized reagents is less than that of traditional methodologies.
6. The specificity and performance parameters of enzymes, such as pH optimum, temperature optimum, thermal stability, and K_m toward a specific substance, may be altered by protein engineering.
7. Enzymes are adaptable (to a varying extent) to change in medium which can vary from pure aqueous to anhydrous organic solvents (10, 11).

It must be emphasized that while immobilized enzymes have advantages, they are not always economical in practice. An example given by Gould and Rocks (12) is worth repeating here to illustrate this point. The use of an open tubular heterogeneous enzyme reactor as a component of an autoanalyzer

method for glucose is economical if at least 140 samples are processed per day. If only 50 samples are analyzed, the immobilized enzyme tube would be 100% more expensive than the pure enzyme. The point of diminishing return is dictated by the shelf life of the immobilized enzyme. Three comments may be added: (1) It may be possible to increase shelf life of the immobilized enzyme by using an alternative immobilization method or matrix. (2) This cost-benefit analysis ignores the fact that even soluble enzymes have limited shelf life. (3) Apart from economic factors, other advantages, including convenience, may favor the use of immobilized enzyme.

5. ENZYME ASSAYS

5.1. Equilibrium Methods

$$S \underset{K_{eq}}{\rightleftharpoons} P$$

An equilibrium method utilizes end-point analysis. Obviously, the greater the extent of conversion, the more accurate the measurement. Because the majority of enzymatic reactions involve two substrates (or one substrate and one coenzyme), it is possible to achieve enhanced conversion by using high concentration of the second reactant X.

$$S + X \rightleftharpoons P + Y$$

An unfavorable equilibrium can also be overcome by chemically trapping one of the products or coupling to a second enzyme. The latter approach has been used most frequently and effectively.

Sufficiently large amounts of enzyme should be used so as to reach equilibrium in a convenient time period. The time period of the reaction is dictated by K_m and V_{max} of the enzyme, as well as by the amount of substrate initially present. Using an immobilized enzyme makes it possible to operate with a huge excess of catalytic capacity, thereby facilitating the establishment of an equilibrium.

5.2. Initial Rate or Kinetic Measurements

The reaction may be monitored as the disappearance of substrate as a function of time $d[S]/d(t)$, or preferably by monitoring the formation of product $d[P]/d(t)$.

6. SEPARATION PROBLEMS IN ENZYMATIC ANALYSIS

Sampling and sample handling are major challenges when applying enzymatic methodologies to the analyses of substances in complex media like biological fluids, food products, and fermentation broth. Traditional enzymology and

enzymatic analysis are usually based on spectrophotometric analysis. This analysis requires optically clear samples with no interfering substances absorbing in the wavelength region where the product is monitored. Extraction of product is then an alternative. If an enzymatic reaction does not give a suitable product for spectrometric monitoring, then an additional enzyme is added to produce a product that is easier to quantify spectrophotometrically.

Immobilization technology makes it possible to reuse enzymes and to restrict them to certain areas of a reaction system. For example, in a flowing system, the product stream is not contaminated with enzymes and the reactions are run in a defined volume element of the total reaction volume.

Immobilizing enzymes also makes it possible to concentrate them in close proximity to certain transducers, called biosensors, to facilitate the monitoring of the reaction. Some of these biosensors operate by principles that make the analyses independent of the optical properties of the sample.

7. IMMOBILIZATION

The various approaches to immobilizing enzymes are broadly classified as follows:

1. Irreversible immobilization
   (a) Covalent coupling
   (b) Entrapment and microencapsulation
2. Reversible immobilization
   (a) Adsorption
   (b) Bioaffinity immobilization

The classification of immobilization methods into two broad categories is especially relevant for the bioanalytical applications of enzymes. Irreversible immobilization implies that the enzyme once attached to the support (soluble or insoluble) cannot be detached without destroying its biological activity. Thus, strong, leakproof binding is an advantage of these methods. In practice, however, this is not always realized. Even the extremely slow rate of leaching is a disadvantage in certain cases, such as during production of pharmaceuticals where contamination by the immobilized catalyst (or ligand) at the ppm level can be disastrous. Nevertheless, irreversible methods often yield adequately stable systems, as evidenced by their continued popularity over the years.

In reversibly immobilized systems, the biocatalyst can be generally detached under gentle conditions which do not normally impair its biological activity. Their advantages over irreversibly immobilized systems can be summarized as follows:

1. No chemical modification of the enzyme is required. The methods utilize gentler conditions as compared to covalent binding and entrapment methods. Entrapment in alginate or K-carragenans is a fairly gentle

method, but the enzyme leaches out quickly unless its size is increased by chemical manipulations. The latter, of course, requires harsher conditions. On the other hand, adding cationic polymers to the gels or using metal ions other than Ca^{2+} to limit the pore size restricts the enzyme inside the gels. Thus, gentler methods for entrapping enzyme may be possible in the near future (13).

2. In real applications (such as in food industries, which are presently the major users of technical grade enzymes), the cost of the support is often higher than that of the enzyme. If the enzyme is inactivated during use, it can be replaced if it is reversibly immobilized. In such a case, the stability of the enzyme does not limit the time period of usefulness of the support of the industrial catalyst.
3. In general, immobilization of the enzyme by adsorption or bioaffinity immobilization can be accomplished rapidly. Consequently, being able to constitute (or reconstitute) the catalyst just prior to use is an advantage.
4. In case of reversible immobilization, minimum amount of the enzyme can be added, whenever required, to obtain optimum conversion rates. This results in considerable economy in cases where costly enzymes are required.
5. Reversible immobilization also can be used for analytical techniques such as affinity sensors or flow injection binding assays (see later in this chapter for a description).

The present state of development in biosensors is at a level where the sensor is used outside the bioreactor. This step has been taken because of sterility problems with the bioreactor and stability problems concerning the biosensor. As long as no sterilizable biosensor is available, most of the applications must be performed outside the fermentor. In such cases, leakage will cause no practical problems, except that more enzyme may be needed.

7.1. Covalent Coupling

Creation of covalent bonds between surface amino acids of the enzyme and an insoluble matrix is perhaps the most frequently exploited method of immobilization. Polar amino acids, which are likely to be present on the protein surface, have structures that lend themselves to the chemical manipulation necessary for immobilization. ε-Amino groups of lysine residues are the most frequently employed points of linkages, though cysteine (via -SH), tyrosine, histidine, aspartic and glutamic acids, tryptophan, and arginine can also be used.

The number of carriers that have been used for enzyme immobilization constitute a long and ever-growing list. Both organic (natural and synthetic) and inorganic carriers have been employed. Some of the more frequently used supports are listed in Table 1.

A number of chemical procedures have been developed for binding enzymes to various matrices. In most of the cases, a matrix is activated and the enzyme is

TABLE I

Frequently Used Supports for Enzyme Immobilization

Organic	Inorganic
Agarose	Controlled pore glass
Dextrans	Ceramics
Cellulose	
Polyacrylamides	
Nylons	
Vinyl polymers	
Chitin	
Chitosan	

Note: See Goldstein and Mannecke (133) for a larger list of support materials.

added. In some cases, a coupling reagent is added in the presence of both enzyme and the matrix. The different coupling techniques have been covered in earlier reviews (14–18).

Directed immobilization, that is, immobilizing the enzyme via groups on certain parts of the molecules that are not part of the active site, is an area where the first successes have been reported. As novel miniaturized biosensors are becoming available, it becomes increasingly important to immobilize the limited amount of biomolecules with a high percentage of retained activity. To direct immobilization to parts of the molecule that are less important for the activity is then an attractive idea. Immobilization of immunoglobulin G via activation of the carbohydrate part of the Fc segment of the molecule does not interfere with the binding properties of the antibody. This coupling is achieved after mild oxidation using periodate to convert the carbohydrates into aldehydes and adding them to hydrazide-derivatized supports (19). Such preparations are now commercially available (BioProbe Int., Tustin, CA, USA).

In most of the covalent coupling methods, it is advisable to protect the active site residues by carrying out the reaction in the presence of substrate analogues (like competitive inhibitors) (20). This simple procedure, although generally not used, does yield an immobilized enzyme of higher activity. Clear effects on retained activity and also concerning sensitivity to allosteric effectors were observed with glutamate dehydrogenase (21) and phosphofructokinase (22). Having ATP present during the immobilization of T_4 DNA ligase yields a threefold higher yield of enzyme activity (23).

7.2. Entrapment

Entrapment involves the capture of the biocatalyst in a three-dimensional network of a polymer. This can be achieved by forming the polymer by a polymerization in the presence of the biocatalyst, or by mixing the biocatalyst and soluble polymer molecules. The latter are cross-linked in a subsequent step. In

this approach, the substrates and products pass freely through the polymeric barrier; the biocatalyst cannot pass through, because it is significantly larger. Obviously, such immobilized enzymes are inappropriate for high molecular weight substrates.

Entrapment of enzymes and cells has played an important role in developing bioprocesses. Applications of entrapment technology to biosensors and bioanalysis have mainly been focused on utilization of cells and, to a smaller extent, on enzymes (24). Combining covalent coupling and entrapment cross-links enzymes and inert protein to form a protein membrane that covers the sensitive part of the electrode tip in bioanalytical applications (25). Entrapping enzyme aggregates is another variation of this methodology (26).

7.3. Adsorption

Adsorbing enzymes onto a variety of matrices is a fairly gentle procedure for immobilizing them. The enzyme is mixed with an insoluble matrix whereupon the enzyme binds to the matrix with weak interactions (H bonds, van der Waals forces, hydrophobic forces, electrostatic forces). In general, the binding is based on a combination of such interactions.

The immobilization of invertase on aluminium hydroxide (2) was one of the earliest reports of adsorption technology. The use of aminoacylase adsorbed on DEAE-Sephadex for producing L-amino acids from a racemic mixture of their corresponding ethyl esters (4) was the first industrial application of an immobilized enzyme system. The basic disadvantage of this convenient technique is that binding is weak and the enzyme slowly leaches out. However, for many purposes, this slow leakage is not an important handicap. Immobilizing enzymes by adsorption has been extensively reviewed (5, 6, 27). Some special approaches are described (1, 28–30).

Coulet et al. (27) describe the immobilization of enzymes on divalent cations chelated to bis(carboxymethyl)amino-derivatized agarose. The linkage through Lewis acid–base-type complexes is reversible, because enzymes could be eluted with EDTA.

Wu and Means (28) immobilized modified enzymes on Octyl-Sepharose in an irreversible fashion. Immobilizing enzymes on tritylagarose is based upon hydrophobic interactions and is reversible (29). Binding to hydrophobic matrices may be either weakened (30) or strengthened by electrostatic interactions (31, 32). Butler (33) has investigated adsorption of different enzymes on esters of allyl and aryl carboxylic acids with cellulosic materials of glass. In the case of phenoxyacetyl cellulose, all ten enzymes that were tested bound strongly. The enzymes are not desorbed with high ionic strength, for example, 1 M ammonium sulfate (salt is known to enhance the hydrophobic interaction) or up to 25–50% of solutions of organic solvents such as ethanol and ethyleneglycol.

Chemical modification may be used to enhance adsorption (1). It was shown that soluble polyanionic derivatives of amyloglucosidase adsorbed on cationic resins much better than the unmodified enzyme.

A novel approach involving chemical modification to enhance adsorption uses perfluoroalkylation of proteins to immobilize them on fluorocarbon matrices (34). In most cases, 10–20% perfluoroalkylation of available amino groups in a protein leads to immobilization. The activity yields after chemical modification are in the range of 66–95%. However, subsequent adsorption led to extensive loss of activity. An alternative procedure involving prior adsorption of perfluoroalkylating reagent onto the surface of fluorocarbon support followed by coupling of enzyme leads to increased retention of biological activity; here, the advantages of reversible immobilization are not retained.

Other recent work shows that the lectin Con A, cross-linked with glutaraldehyde, binds to DEAE-cellulose, irreversibly. The bound lectin possesses adequate biological activity with respect to binding to glycoprotein enzymes (35). Trypsin modified by pyromellitic anhydride binds much better to DEAE-cellulose as compared to native enzyme (M. N. Gupta, unpublished results).

7.4. Stability of Immobilized Proteins

Proteins are known to get denatured by exposure to heat, chemical denaturants, extreme pH, and organic solvents. Although immobilization imparts stability, even in the most favorable cases, this stability is not unlimited (36). In general, the denaturation/inactivation of immobilized proteins may be broadly divided into two kinds of processes (37): reversible and irreversible.

Reversible denaturation involves the unfolding of the native conformation of the protein and aggregation. Irreversible denaturation involves some covalent modifications. The nature of these modifications depends on the inactivation process. Perhaps, the best understood phenomenon in this regard is thermal inactivation which results in chemical changes that cause irreversible damage to protein. These changes include deamidation of asparagine residues, disulfide exchange reactions, and peptide bond cleavage of aspartic acid residues (38).

How does immobilization stabilize a protein? First, it reduces the intermolecular interaction, thus abolishing aggregation (38). Second, it minimizes the unfolding of the protein molecule by multipoint attachment to the matrix. Thus, the number of attachment bonds between the enzyme and the matrix correlates well with the enhancement in stability achieved as a result of immobilization (39). Mozhaev et al. (39) note that this stabilization cannot be pushed beyond a certain limit because irreversible denaturation is always possible.

These considerations are important in the context of analytical applications since they dictate the overall operational half-life of the immobilized system. In fact, an immobilized enzyme is particularly vulnerable to deactivation because it is unlikely to be used for monitoring a pure substance. Exposure to a physiological fluid or fermentor medium exposes the catalyst to inhibitors or extraneous matter. For example, the half-life of the immobilized lactase decreased from 89 days to 7 days when the substrate changed from 5% lactose solution to acid whey (40).

8. ANTIBODY–ENZYME INTERACTIONS

Antibodies raised against enzymes (antigens) can be used for the reversible immobilization of enzymes. The binding of antibodies to antigens is reversible and the binding constants vary from below 10^5 mol^{-1} to above 10^{12} mol^{-1} (41). These remarkable binding constants are possible because of the multivalent nature of both antibody and antigen molecules. In the case of polyclonal antibodies, the average affinity constant is generally defined as the reciprocal of the free antigen concentration at equilibrium when half of the total antigen binding sites are occupied (42). It is now known that the distribution of antibody affinities in an antiserum to an antigenic determinant is non-Gaussian (42). Thus, it is only meaningful to assign a specific number to the binding constant between an antigen and its monoclonal antibody (41). Therefore, monoclonal antibodies constitute the most specific affinity ligand for the purpose of reversible immobilization of enzymes.

The purifications of several mouse monoclonal antibodies against carboxypeptidase A were described by Solomon et al. (43). The binding constants of these antibodies for the enzyme were of the order of 10^6 M^{-1}. They varied in their capacity to inhibit the esterase and peptidase activity of the enzyme. Some inhibited only peptidase activity, some inhibited esterase activity, while some monoclonals inhibited both activities. Solomon et al. also obtained antibodies that did not affect the activity of the enzymes upon binding. Subsequently (44), a monoclonal antibody with a higher binding constant (ca. 10^9 M^{-1}) was produced that did not affect any of the catalytic activities of the enzyme. Using this antibody made it possible to reversibly immobilize carboxypeptidase A on this antibody coupled to Eupergit C (covalently) or Sepharose–protein A (noncovalently). This work illustrates the superiority of reversible immobilization over covalent immobilization in as much as the covalent binding of carboxypeptidase A to Eupergit C either gave no enzyme activity or gave much reduced activity (30–40% residual activity). The enzyme immobilized on antibody columns also displayed enhanced stability toward incubation at 50 °C and exposure to acidic conditions (pH 4.5–7.5) (45).

A recent paper by Liapis et al. (46) suggested that the Langmuir model could provide a satisfactory correlation of the equilibrium experimental data of the adsorption of lysozyme onto its antibody immobilized on nonporous silica particles.

Selection of antibody is a critical point. Traditionally, a high affinity has been preferred, since it gives high sensitivity in the assay. Assays normally are dead-end binding reactions.

When it comes to processes involving reuse of antibodies, for example, immunoaffinity purification or reversible immobilization via antigen–antibody interactions, slightly different criteria may be set up when selecting the antibody. A firm binding is still a desirable property, but it must be combined with dissociation conditions that do not destroy the antibody. This often leads to selection of antibody preparations with lower affinities.

9. ENZYME STABILIZATION BY ANTIBODIES

Knowing that peptides and amines confer thermal stability on enzymes from certain thermophilic organisms (47–49) led some workers to examine protein stabilization by antibodies. It was found that the presence of specific polyclonal antibodies stabilize several enzymes (50, 51). In addition, not only did antibodies increase the thermostability of α-amylase, glucoamylase, and subtilisin, but some stability toward acid denaturation, oxidizing agent, and organic solvent exposure was increased in specific cases (52, 53).

10. LECTIN–GLYCOENZYME INTERACTIONS

Lectins are carbohydrate binding proteins of nonimmune origin that agglutinate cells or precipitate polysaccharides or glycoconjugates. These molecules were first discovered in plant seeds, but are now known to occur in microbial, avian, and mammalian sources as well. A large number of them have been purified and are commercially available. Most are glycoproteins although some are simple proteins. Concanavalin A and wheat germ agglutinin are well-known examples of the latter category.

Detecting and assaying lectins are made possible by the ability of lectins to agglutinate red blood cells. The specificity of lectins is in terms of the simple sugars that inhibit the agglutination assay. Some lectins, of course, recognize somewhat more complex carbohydrate structures. Table 2 gives a list of some lectins to illustrate the features of this class of proteins.

The principal application of lectins in bioanalytical systems involves the reversible immobilization of glucose oxidase, invertase, and peroxidase on Con A–Sepharose. Such lectin-based affinity media have also been utilized for immobilization of glycoenzymes. Woodward (18) shows that cellobiase is not desorbed by its substrate cellobiose and product glucose from the support matrix.

TABLE 2

Some Well-Characterized Lectins

Lectin	Sugar Specificity
Concanavalin A	Mannose/glucose
Peanut lectin	Galactose
Soybean lectin	*N*-Acetyl galactosamine
Potato lectin	*N*-Acetyl glucosamine
Horseshoe crab lectin	Sialic acid
Wheat germ lectin	*N*-Acetyl glucosamine
Jecalin	Galactose

Note: For a more comprehensive list and further information see Goldstein and Poretz (134).

11. AFFINITY-DIRECTED IMMOBILIZATION

Turkova et al. (54) irreversibly immobilized carboxypeptidase Y on a Con A matrix by treating the enzyme–lectin complex with glutaraldehyde. A variation of the approach exploiting lectin–carbohydrate interactions for affinity immobilization is described by Khare and Gupta (55). In this work, a conjugate of Con A with *Escherichia coli* β-galactosidase (which is not a glycoprotein) is immobilized on Sephadex matrix exploiting the affinity of the latter toward Con A. The immobilized preparation was used for hydrolysis of lactose.

The use of lectins and glycosylated enzymes in bioanalytical systems is depicted schematically in Fig. 1. These systems are especially useful when assays require labile and expensive enzymes, because small amounts of enzymes can be added and they need to remain active for only short time periods. Most enzyme-based assays are based on the use of stable enzymes and that has restricted the development of new assays. In principle, this concept would also be useful for cells, but because of multipoint attachment it may be cumbersome to regenerate the sorbent.

12. USE OF GENETICALLY FUSED AFFINITY TAGS

Recombinant DNA technology makes it possible to construct fusion proteins (56), that is, proteins that result from the expression of fused genes. Fusing or joining two or more distinct genes by genetic engineering techniques produces a coding sequence that is transcribed as a single unit. The idea has been utilized for protein separations, production of cell-specific cytotoxic agents, and creation of

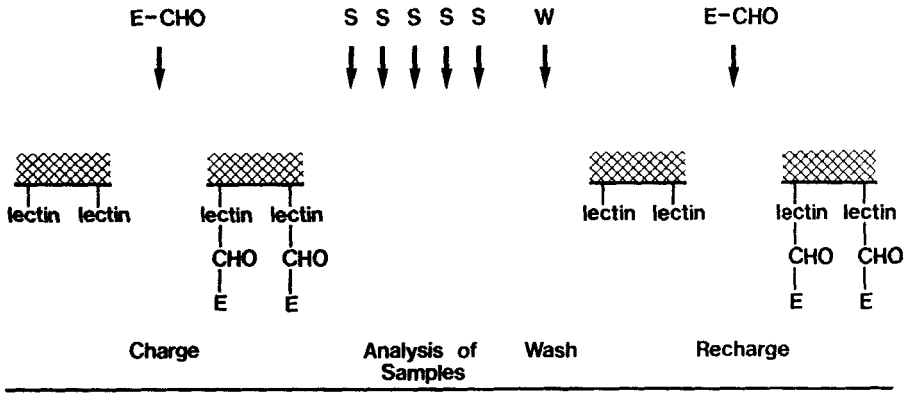

Fig. 1. An assay procedure using reversible immobilization of glycoenzymes to covalently bound lectins. The starting position involves the immobilized lectins. The enzyme that is a glycoprotein (E–CHO) is introduced as a pulse and binds to the lectin. Assays are performed (arrows with S) and when the analyses are over or the enzyme is denatured, washing takes place (arrow with W). This brings the immobilized lectin back to starting position, and either fresh enzyme or a new glycoenzyme may be introduced.

hybrid enzymes with novel characteristics (57–60). Because one of the proteins in the hybrid can be the partner of an affinity pair with a suitable binding constant, it is possible to create immobilized enzyme systems (61). In fact, the technique is quite versatile and, in principle, can be used to immobilize enzymes via ionic, hydrophobic, or metal chelate interactions as well. Using the gene fusion technique makes it possible to attach an affinity tag to the desired protein, which in turn permits the immobilization of the protein to a matrix that possesses the affinity tag.

Sassenfeld (62) recently discussed various affinity tags. Their suitability for application for affinity immobilization is described below.

12.1. Enzyme Tags

Using enzyme tags makes it easier to exploit the availability of a large number of affinity media available for purification of enzymes. Thus, a fusion protein can be constructed with an enzyme (tag) that binds to the affinity media. Enzyme tags that have been used are β-galactosidase (56, 57, 63–68), chloramphenicol acetyltransferase (69, 70), alkaline phosphatase (58), and glutathione-*S*-transferase (71). β-Galactosidase is the most popular enzyme tag. This method holds good promise for immobilizing enzymes in bioanalytical systems.

12.2. Affinity Tags

A biomolecule with specificity for a special molecule (which is used as a ligand on an immobilization matrix) is modified by an affinity tag. The tag may be a synthetic antigen, such as Flag, which consists of eight amino acids and binds to an immobilized monoclonal antibody (72). The interaction of Flag with the monoclonal antibody is Ca^{2+}-dependent and reversible at low pH. These features constitute an undesirable constraint when applying this fusion to affinity immobilize some enzymes.

The affinity tag may be a member of a natural affinity pair. Protein A is an example of this category (73, 74) since it binds to the constant region of immunoglobulins. Another approach exploits carbohydrate binding proteins (75–77). Only cellulose and maltose binding proteins have been used to date. However, a large number of carbohydrate binding proteins are available (78), including lectins. The immobilization of β-galactosidase fused to cellulose binding protein has already been successfully performed (76). The cellulose binding domain of cellulases has been characterized. There are domains suitable for fusion with the N-terminus and another for the C-terminus of the protein. Use of these affinity tags thus offers great flexibility. Varying the binding strength to cellulose by fusing various domains is especially valuable from the viewpoint of reversible immobilization (75)

12.3. Other Tags

Several other tags are available, which employ electrostatic or hydrophobic interactions or metal chelate formation including polyarginine (79), polyphenylalanine or polycysteine (80), and dipeptide His–Trp (81).

It may appear that the use of genetically fused affinity tags could eventually replace existing methods of reversible immobilization. However, while it is undoubtedly the most recent and a powerful tool for reversible immobilization, it is not without its limitations. The organisms commonly used for expression of heterologous proteins are *E. coli* and *Saccharomyces cerevisiae.* When the protein expressed in *E. coli* are not secreted they frequently form insoluble protein aggregates (inclusion bodies) which are believed to form due to incorrect folding of polypeptides. Such inclusion bodies are generally solubilized in urea or guanidinium hydrochloride. Thus, any enzyme that is susceptible to irreversible denaturation in the presence of these denaturants cannot be used either as a tag or immobilized via a tag. Perhaps recent work on enzyme-catalyzed protein folding (82) may lead to a greater understanding about the formation of inclusion bodies and the development of ways to prevent their formation.

Another problem that may arise is that the protein fusion may lead to inactivation of the enzyme. The destabilization of β-galactosidase when using a polyphenylalanine tag is an illustrative example (80). There may also be interference between SH groups on the protein and cysteine groups on the fused affinity tail.

Despite these problems an increased use of genetically fused affinity tags for bioaffinity immobilization is anticipated. The main advantages of this approach over conventional bioaffinity immobilization are the following:

1. Because the catalytic component and binding component are different, binding to the matrix should not affect the catalyst.
2. The binding component automatically acts as spacer, which minimizes unfavorable interactions of the catalyst with the support matrix.
3. The affinity matrices are often quite costly since they employ biochemicals such as lectins and monoclonal antibodies. Development of less expensive technology for production of fusion proteins may be an economical alternative.

13. WHAT IS POSSIBLE IN REVERSIBLE IMMOBILIZATION

Reversible immobilization is a relatively new approach for immobilizing the catalytic component. Table 3 indicates the various interactions that could be explored for this purpose. Their usefulness is largely unexplored for reversible immobilization and individual researchers should consider their individual requirements and constraints before adopting any one of them for their purposes.

14. DOPED ELECTRODES

Assays involving oxidases are limited by the low solubility of oxygen in the medium (83) and the harmful effects of one of the products, hydrogen peroxide,

TABLE 3

Examples of Affinity Pairs to Be Used for Reversible Affinity Immobilization

Ligands	Proteins Reported to Have Affinity Ligand
Heparin	Growth factors, coagulation factors, lipoproteins, DNA polymerase, DNA ligase, RNA polymerases, restriction endonucleases, polynucleotide kinase, lipoprotein lipase, hepatic triglyceride lipase, reverse transcriptase, hyaluronidase, neuraminidase, trypsin, pepsin, fumarase, lectin from chicken liver and embryonic chicken muscle, platelet-secreted antiheparin proteins, platelet-endoglycosidase
Cibacron blue F3GA	Human plasma binding protein for vitamin D, human fibroblast interferon, glutamine synthetase, T4 DNA ligase, aspartate carbamoyltransferase
Boronic acid	Glycoenzymes, ribonucleosidases
Protein A	Immunoglobulins
p-Aminobenzamidine	Trypsin, enterokinases, tyrosinase, urokinase
p-Amino benzyl phosphonic acid	Alkaline and acid phosphatases
Avidin	Biotin-containing enzymes and biotinylated enzymes
Biotin	Avidin and biotin binding proteins
2-Iminobiotin and diaminobiotin	Same as biotin but affinity complexes dissociate under more gentle conditions
Streptavidin	Same as avidin but is not a glycoprotein, and reduced nonspecific binding
p-Aminophenyl-β-D-thiogalactophyanoside or aminobenzyl-1-thio-β-D-galactopyranoside	β-Galactosidases
Sugar and sugar derivatives	Lectins, carbohydrolases
Lectins	Glycoenzymes
Protease inhibitors	Proteases
Protamine	Phosphoprotein phosphatases and malate thiokinase
Glycyl-L-tyrosyl-azo-benzyl succinic acid	Carboxypeptidases
Iminodiacetic acid	Phosphotyrosyl-protein phosphatase, protease inhibitor, NADH: nitrate reductase
L-Alanyl-L-alanyl-L-alanine	Elastases
N-Acetyl-DL-homocysteine	Isocitrate dehydrogenase; in general, useful for reversible covalent immobilization approach
p-Chloromercuribenzoate	Reversible covalent immobilization of -SH-containing proteins; several such other ligands are also commercially available

on the enzymes by one of the products, hydrogen peroxide. When enzyme electrodes are operated in a continuous mode, the lifetime of an enzyme decreases dramatically when compared to that of an enzyme used in an intermittent mode. The decrease is apparently due to the peroxide generated. Several approaches have been tried to eliminate this problem. Co-immobilization with catalase increases the life-span for the enzyme and the linear concentration range for analysis (84) because the hydrogen peroxide is decomposed by catalase to form oxygen.

A recent successful approach uses mediators like ferrocene, which reoxidizes the $FADH_2$ in the glucose oxidase molecule without forming hydrogen peroxide (85). Using ferrocene results in a more stable electrode system and a larger dynamic concentration range. The ferrocene molecule is immobilized by adsorption to graphite or other carbon electrodes onto which the enzyme can be deposited.

Electrodes doped with mediators are also successful in analyses using NAD (P)-dependent dehydrogenases (86–88). In these cases, the mediator is firmly adsorbed to the electrode. The cofactor is oxidized by the mediator, which becomes reduced. The mediator is reoxidized by an electrochemical process on the electrode. This technology makes it possible to reduce the amount of cofactor needed, for example, in flow injection analysis and also eliminates the need for enzymatic regeneration systems. A further successful development uses a carbon paste chemically modified with a dehydrogenase, the coenzyme, and a phenoxazine mediator. This complex structure is then coated with a polyester sulfonic acid cation exchanger (86). The mediators used are of aromatic polycyclic structure and are firmly bound to graphite or other carbon electrodes (Fig. 2) (89).

15. SOLUBLE POLYMER-BOUND BIOMOLECULES IN ANALYSIS

In some respects there are advantages operating with biomolecules in free solution. Steric limitations do not interfere with the reactions, and equilibria are

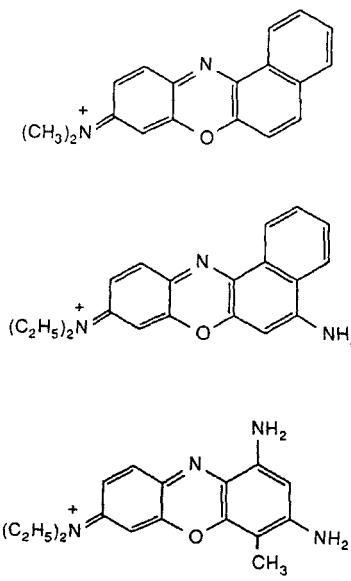

Fig. 2. Structural formulae of Meldola blue (*top*), Nile blue (*middle*) and brilliant crysyl blue (*bottom*). All three compounds have been used in conjunction with chemically modified electrodes. (After Persson (89).)

quickly achieved in binding reactions. On the other hand, soluble reactants are normally lost from the system.

Using membrane reactors makes it possible to use enzyme reactions in free solution with retained activity. This approach has not attracted much interest, partly because the benefits over the immobilized state are minimal. However, for coenzyme-dependent enzymes, there is a need for new technology (one such new development is the doped electrodes discussed above). The use of co-enzyme-dependent enzymes in enzyme technology has been very restricted. The small, but expensive, coenzyme molecules causes problems, since they must be retained and regenerated in the system. The coenzyme must be recycled many times in order not to ruin the economy of the process. Dehydrogenases constitute a major group of catalysts with a great potential in enzyme technology. But the use for the cofactor NAD(P) has limited the use of dehydrogenases.

Operating with enzymes as well as coenzymes firmly immobilized does not allow molecular mobility, and thus no reaction takes place. Efforts to co-entrap the entities reveals that a rapid leakage occurs with small coenzyme molecules. However, when coimmobilizing the enzyme(s) and a polymer-modified coenzyme, a stable activity could be maintained without adding external cofactor (90). Experiences with biocatalytic processes using polymer-bound coenzymes together with enzymes in free solution retained behind a suitable ultrafilter membrane are successful (91, 92). In a later improvement of this approach, Kulbe demonstrates the possibility of using native coenzymes retained behind a charged membrane (93).

Using soluble reactants retained behind a membrane forms the basis of the design of an optical biosensor for glucose. A hollow fiber is mounted at the end of an optical fiber, the free end of the hollow fiber is plugged, and Con A is immobilized on the inner surface of the hollow fiber. Fluorescein-labeled dextran binds to the Con A. When free glucose enters the hollow fiber, a competition occurs between the labeled dextran and the free glucose for binding to Con A. Some fluorescein-labeled dextran is liberated and passes out into the lightpath where the fluorescent groups are excited by light from the fiber. The emitted light is registered either via the same fiber or through a parallel fiber (94, 95). This type of a competitive binding assay can also be applied to other binding structures and metabolites.

16. PROTEINS CONFINED IN LIQUID–LIQUID EXTRACTION SYSTEMS

When enzymes are added to a mixture of two incompatible aqueous polymer solutions, they often partition to one of the phases (96, 97). Mixing produces a fine emulsion with droplets of one of the phases distributed in the other continuous phase. The enzyme that partitions into a droplet may be regarded as being temporarily immobilized (98). Mass transfer across the droplet interface is facilitated compared to traditional immobilized systems. Such systems are

especially suitable for analyses of macromolecular and particulate substrates (99).

Binding assays performed in aqueous two-phase systems offer certain advantages. Provided the different reactants favor different phases, the complex between them will change the partition behavior of either of the reactants (100–102). To achieve partitioning of the reactants into different phases, chemical modification may be used. In partition affinity ligand assay (PALA), immunochemical binding as well as other specific interactions have been exploited. The general principle behind this assay procedure is shown in Fig. 3. A wide variety of target molecules have been analyzed by applying this technique (Table 4).

Assays of enzymatic activity of polymer degrading enzymes are often difficult to perform using traditionally immobilized systems. Using a liquid–liquid phase system makes it easier to establish contact between the catalyst and the substrate. Products may then partition differently as compared to the substrate. This forms the basis for the assay of dextranase using a dye substrate, blue dextran. The liberated color is preferentially extracted into the more hydrophobic top phase (103). Adding a ferrofluid that is a submicron magnetic preparation to an aqueous two-phase extraction system makes it possible to substantially facilitate phase separation by magnetic force (104).

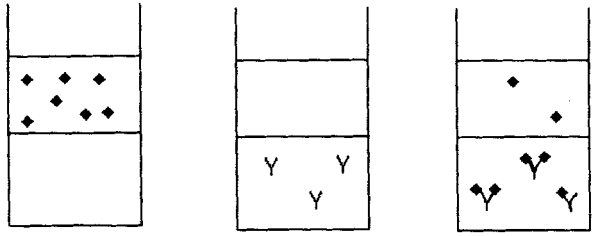

Fig. 3. A direct binding assay between antigen and antibody. The antigen partitions into the top phase (*left*) and the antibody partitions into the bottom phase (*middle*). Binding is observed as a displacement of antigen from the top phase to the bottom phase (*right*) (From Ling and Mattiasson (102).)

TABLE 4

Partition Affinity Ligand Assay: Immunoassays of Different Antigens

Type of Antigen	Antigen	Concentration Range
Hydrophobic hapten	Digoxin	1–8 nM
	T_3	1–6 nM
	T_4	50–200 nM
Protein	β_2-Microglobulin	3–96 μg/L
Cells	Staphylococci	10^6–10^7
	Streptococci	2.5×10^3–10^4

Source: Adapted from Ling and Mattiasson (102).

17. PROTEIN IMMOBILIZATION ON MAGNETIC SUPPORTS

An immobilized enzyme reagent can be recovered and reused. This separation normally involves centrifugation or filtration. A simpler and more convenient alternative (105) uses a magnetic field to separate an immobilized preparation that has been rendered magnetic. This approach is especially valuable if the assay medium contains other particulate matter (in which both filtration and centrifugation would not be useful in the separation of enzyme) or materials that would clog filters. Physiological fluids, and environmental samples and fluids, and fermenter products are a few examples where such a situation is common.

Various techniques may be used to convert enzymes into magnetic reagents:

1. Adsorption of proteins to iron oxides followed by cross-linking with glutaraldehyde (106, 107).
2. Human serum albumin coupled to Sepharose-entrapped iron oxide particles from a ferrofluid (108).
3. Entrapment of carbonyl-iron in starch and coupling of BSA to this matrix using cyanogen bromide (109).

Coupling proteins (by covalent methods) to magnetic supports is the most common approach (105). Various classes of proteins, such as lectins (110) and antibodies (111), and a large number of enzymes including invertase (112, 113), papain (106, 114) α-chymotrypsin (114, 115), β-galactosidase (114), adenylate kinase (112), acetate kinase (112), and horseradish peroxidase (112), have been immobilized. In fact, such supports are now commercially available, for example, Enzacryl FEO-M (available from Aldrich Chemical Company, USA) and Dyna (sold by Dynal, Norway).

One of the first applications of magnetic separation in bioanalysis involves immunoassays (for a review, see Pour Carzaneh et al. (111). Here one specie of the immune couple is immobilized onto the magnetic particle and magnetic separation is performed after formation of an antigen–antibody complex. After proper washing, dissociation of the complex was performed before reading the outcome from the analysis as the amount of bound and then liberated material.

The versatility of magnetic enzyme preparations in bioanalysis can be illustrated with a recent example. Miyabayashi and Mattiasson (116) describe a novel type of an enzyme electrode for determination of glucose. Glucose oxidase is coupled to magnetic beads. Attaching the tip of a Clark-type oxygen electrode to an electromagnet makes it possible to obtain a homogeneous distribution of these magnetic "enzyme particles" at the tip of the electrode. Thus, the biocatalyst component can be added or removed as necessary; that is, the "enzyme electrode" can be reconstituted just before use. As stated above, magnetic separation is a powerful technique when dealing with particulate matter. An interesting application (which lies outside the central scope of this review) is separation and analysis of cells in complex media. Technology for cell separation has been developed with special emphasis on bone marrow cells. The technology developed in such studies would be very useful to implement in cell counting and cell analysis.

As an example, the use of nanoparticles onto which suitable ligands were bound is described (117). In most cases when magnetic affinity materials are used for separation, the magnetic beads are large in comparison to the structures to be separated. Such an arrangement puts some constraints on the separation system in the sense that upon mixing severe shear forces may disturb the affinity interaction. The use of nanoparticles eliminates that problem, since the structure to be separated is the major component of the affinity complex, and the affinity material constitutes only a minor part (Fig. 4).

ENZYMATIC ANALYSIS IN WATER-POOR MEDIA

The development of enzyme-catalyzed processes in organic solvents makes it possible to perform enzymatic analysis in organic solvents. Earlier work involved the addition of moderate amounts of solvents to improve substrate solubility, but the new trend is to operate in almost water-free conditions. The selection of reaction parameters is important. Thus, it is necessary to optimize the solvent (118, 119) as well as the enzyme support (120). The polarity of the solvent is also important; the more polar the solvent, the less stable the enzyme (119). Thus, extremely hydrophobic solvents are useful, provided the substrates and products are soluble. The choice of support is governed by its tendency to attract minute amounts of water present in the system. The supports are characterized with regard to their aquaphilicity: There is an inverse correlation between aquaphilicity and catalytic activity of the adsorbed enzyme (121).

Thus far, few analytical applications of enzymes in a nonaqueous solvent are available. One example is the use of oxidases to oxidize hydrophobic substrates,

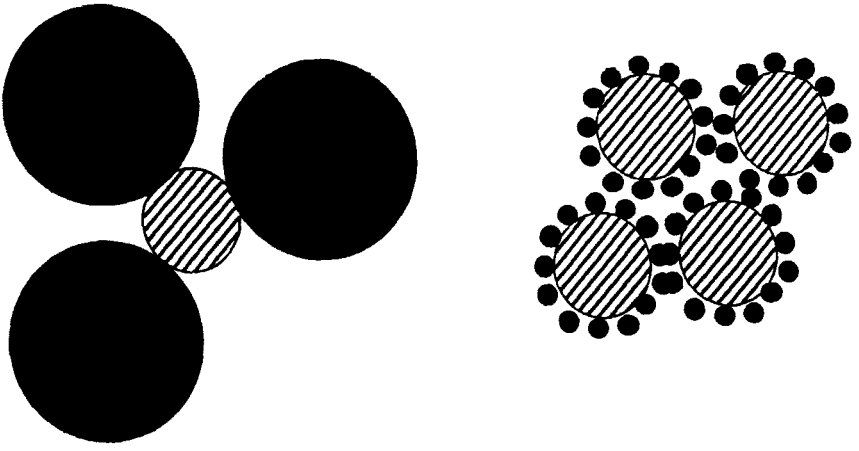

Fig. 4. Magnetic cell separation. (*Left*) Traditional method using large magnetic particles to bind to the cells. (*Right*) The use of magnetic nanoparticles that interact with the cell surface and thereby form large aggregates that can be collected with a magnetic trap.

such as cholesterol (122) and *p*-cresol (123). An additional advantage of using these oxidases in a nonaqueous solvent, besides the solubility of the organic molecule, is the improved supply of oxygen.

Under the section on magnetic immobilized preparations we described the use of a polarographic oxygen electrode onto which magnetic particles carrying glucose oxidase could be reversibly immobilized. The same technology, using a magnetic charging of the enzyme electrode, has been utilized in bioorganic analysis (124). A potentiometric electrode is used, onto which a dry magnetic preparation of chymotrypsin is attached by means of an electromagnetic field to monitor ester synthesis. This approach makes it possible to add and/or remove enzyme without having to interfere with water. The magnetic enzyme preparations may be dry and they are suspended in a dry organic solvent before being trapped on the tip of the electrode. The enzymatic activity during both conventional bioconversion processes in organic solvents and when being applied to biosensing is severely influenced by variations in the water content. Effective control of the water activity in the medium is a prerequisite in the successful use of enzyme based sensors in organic solvents.

A unique area of enzyme analysis is the use of enzyme reactions as a time-temperature integrator to monitor storage of frozen products. The general principle is based on an enzymatic reaction that generates a colored product. At low temperatures no reaction should take place, but if the temperature increases then enzymatic activity increases and colored products are formed. The indicator is fixed to a product that needs cold storage, and a color develops if the product is exposed to high temperatures for a time period long enough to affect the enzyme.

Two different types of enzymatic time–temperature integrators are described. The first, under the tradename of I-point, is based on a lipase-catalyzed hydrolysis reaction (125). The lipase is stored in a nonaqueous environment containing glycerol. The indicator contains two components that are mixed when the indicator is activated. The operating principle is as follows: Upon activation, two volumes of reagents are mixed with each other. Lipase is thereby exposed to its substrate, here a triglyceride. At low temperatures there will be almost no hydrolytic reaction. As the temperature increases, hydrolysis accelerates and protons are liberated. A pH indicator is dissolved in the system. The indicator is selected to shift color after a certain amount of acid has been liberated by the enzyme-catalyzed process. Since the catalytic activity is influenced both by temperature and time, this indicator strip is said to be a time–temperature integrator.

The second time–temperature indicator is based on the use of horseradish peroxidase in liquid and solid paraffin (126). The enzyme is deposited onto nonporous glass beads and mixed with melted paraffin containing the substrate. The suspension is mixed well and quickly cooled in a dry ice/acetone bath. When the enzyme is stored in solid paraffin, the activity is extremely slow. But when the temperature increases, the paraffin may melt and thereby make the enzyme reaction a millionfold faster than in the solid hydrocarbon phase. This time–temperature integrator is based on the same concept as the I-point, but here the

reaction medium undergoes a transition from solid to liquid, thereby facilitating the reaction. The enzyme-catalyzed process is a chromogenic reaction. The indicator thus shifts from colorless to colored.

19. VARIOUS FORMS OF ENZYME-BASED ANALYTICAL DEVICES

Immobilizing enzymes makes it possible to integrate them into various forms of enzyme-based analytical devices. Most of these have unique applications and advantages that are not possible with soluble enzyme-based systems. Biosensors, as these biocatalyst based devices are called, constitute a frontier area of biotechnology (127, 128).

The above mentioned review (127) also contains a discussion of the ways in which immobilized enzymes can be integrated into various kinds of transducers that convert the output from the biosensing element into an electronic signal and the range of substances that have been analyzed with such biosensors.

20. APPLICATIONS

20.1. Flow Injection Antigen–Antibody Binding Assay

The binding assays that are performed in this flow injection system may be either competitive or sandwich type. An assay for the serum protein transferrin using a competitive ELISA (129) illustrates the principle (Fig. 5). An immunoaffinity purified polyclonal antiserum raised in rabbits against human transferrin is the binder. The immobilized antibody is packed in a small column (100–200 μL), which is placed in a continuous flow of buffer. The experimental setup is shown in Fig. 6.

Equilibration buffer is pumped over the bed at a constant flow rate (0.7 mL/min) so as not to cause compression of the bed. A mixture of transferrin and HRP-labeled transferrin is then introduced via a sample loop (200 μL total volume). After a short pulse of buffer to remove enzyme label not properly bound, substrate (0.26 mM ABTS and 0.002% hydrogen peroxide) for the enzyme is introduced in a short pulse (200 μL total volume). The continuous flow is passed through a small flow cell (30 μL) in a spectrophotometer. After completion of the assay, a pulse of glycine–HCl, pH 2.2, is introduced to reverse the antigen–antibody complex and to ready the antibody column for another assay cycle. Total time for one assay cycle is in the range of 6–7 min. For a control, the assay cycle is repeated with just enzyme-labeled antigen and with mixtures of the same amount of labeled antigen with varying mixtures of native antigen (Fig. 6).

As stated initially, the flow system offers well-controlled reaction conditions for binding, washing, incubation with substrate, and data collection. The flow system is based on a constant flow rate and in the assay cycle it offers a very short

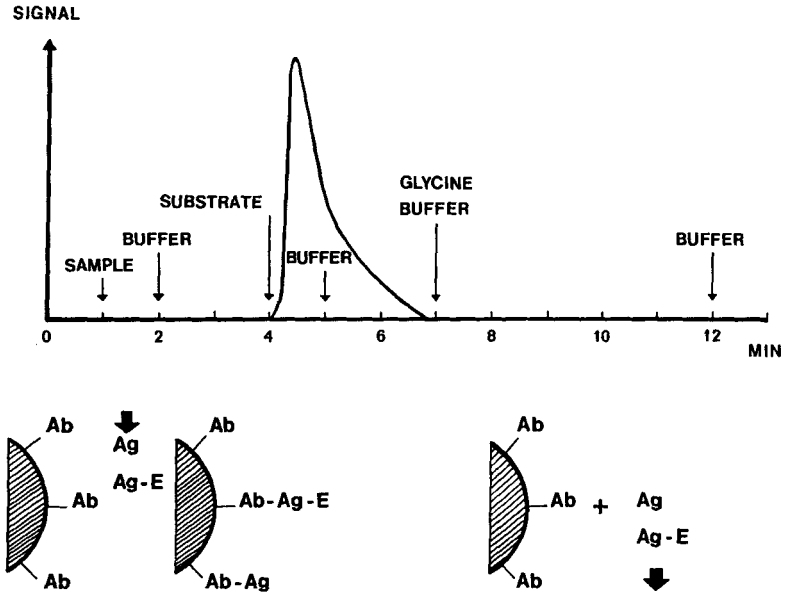

Fig. 5. An assay cycle using the flow-ELISA principle. The sample supplied with a fixed amount of enzyme-labeled antigen is introduced at the arrow "sample" into the continuous flow stream. Buffer is fed for a short period before substrate for the marker enzyme is given as a pulse. The system is rinsed by a pulse of glycine buffer, pH 2.2. After reconditioning, the system is ready for a new assay. (After Mattiasson et al. (135).)

time for contact between antigen in the sample pulse and the immobilized antigen. The system thus operates far from equilibrium with regard to the immunological binding reaction. Furthermore, since washing buffer is applied through the flow system, the washing procedure is kept under very strict control.

Flow injection systems are built around high-quality pumps. By utilizing such equipment in combination with sample loops for injection of exact volumes of samples, washing buffers, substrate, and so on, it is possible to substantially reduce the experimental errors that usually go with ELISA methods. One example of the unique reaction conditions is that the use of a well-defined contact time between the antigen-containing sample and the antibody column has made it possible to operate far from equilibrium. There is per se no characteristic of the ELISA responsible for poor reproducibility; rather, all the different manually operated experimental steps contribute to the variations. By eliminating these to a large extent, the reproducibility is improved. A limiting factor is that upon reusing the immobilized antibody, slow denaturation in the dissociation step may occur, and this will lead to a reduced capacity of the subsequent analysis. However, procedures have been developed to deal with this problem. Non-

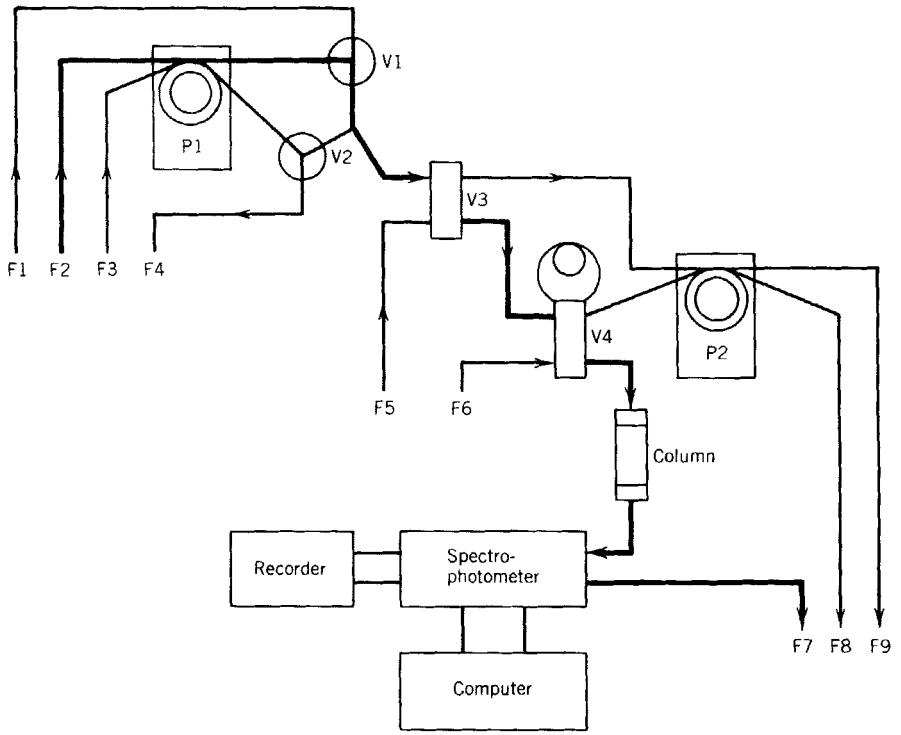

Fig. 6. The experimental setup for performing automated flow injection–ELISA measurements. F1, excess flow of main buffer; F2, main buffer flow; F3, washing buffer; F4, excess flow of washing buffer; F5, substrate solution; F6, sample solution; F7–F9, waste streams; P1 and P2, peristaltic pumps; V1 and V2, 3-way valves; V3 and V4, injector valves. The computer logs and evaluates the data and controls the flow through the whole system by controlling valves and pumps. (From Nilsson et al. (143).

equilibrium immunoassays can be handled due to the unique reproducibility in the reaction conditions applied. These factors help to reduce variation between repetitive assays of a sample to within 2%, far better than in conventional ELISAs.

One characteristic of these assays is that the calibration curve obtained for such an immunosorbent column is valid throughout the lifespan of the preparation. One needs only to compensate for decrease in capacity. This means that a value obtained with just enzyme-labeled antigen passed through the column intermittently, and subsequent reading is then compared to that calibration value.

Assays according to this procedure are fully automatized for process monitoring and control, both in cell culture and in downstream processing (7, 8). Table 5 presents some of the systems available today.

TABLE 5

Examples of Analysis Performed and Detectors Used in Flow Injection Binding Assays

Metabolite	Detector	Comment	Reference
Human serum albumin	Calorimeter	Macromolecular target	135
Gentamicin	Spectrophotometer	Hapten	136
Insulin	Polarographic oxygen electrode	The first immuno electrode	137
Proinsulin	Calorimeter	Automated assay	138
Methyl-α-manno-pyranoside	Polarographic oxygen electrode	Lectin–carbohydrate interactions	139
Transferrin	Spectrophotometer	Flow injection equipment used	129
Methyl-α-manno-pyranoside	Spectrophotometer	In-line substrate administration	140
Immunoglobulin G	Electrochemical detector	Sandwich assay	141

20.2. Reversible Immobilization of Enzymes via Biospecific Interaction with Lectins

To illustrate this principle, we have chosen a lectin–glycoprotein system (130). The glycoenzymes glucose oxidase and peroxidase are bound to immobilized Concanavalin A or lentil lectin coupled to Sepharose. The immobilized lectin is packed in a small column inside a simple flow calorimeter. A continuous buffer stream (flow rate 0.75 mL/min) is pumped through a small column, at the outlet of which is placed a thermistor. This unit is well insulated from the surroundings.

The glycoenzyme is introduced into the flow stream and allowed to pass through the column. Enzyme is trapped by the lectin–carbohydrate interactions. The immobilized enzyme is now ready to be used in analyses. Pulses of substrate (1-min duration) are passed through the column and the reaction heat is registered by the thermistor. When the enzyme denatures or one wishes, to change to another enzyme, the affinity bound enzyme can be removed from the column by a simple affinity displacement using either a pulse of α-methyl-D-mannopyranoside or a pulse of glycine–HCl, pH 2.2, that will dissociate the interaction. After being washed with perfusion buffer, the lectin column is ready for a new cycle.

20.3. Enzyme Electrode with Magnetic Beads Carrying the Enzyme Activity

Replacing the enzyme component of an enzyme electrode in a convenient and reproducible way is often desirable. The use of magnetic beads carrying the immobilized enzymes in combination with an electrode placed in a homo-

geneous electromagnetic field that can be switched on and off often meets these demands (116).

Magnetic beads are charged with Concanavalin A by covalent coupling and are then allowed to react with the glycoprotein, glucose oxidase. A Clark-type oxygen electrode is placed in a solenoid that is adjusted to give a constant magnetic field at the tip of the electrode. Increasing the current and thereby the field strength provides a more homogeneous distribution of the magnetic particles containing Con-A-immobilized glucose oxidase as monitored by eye.

The electrode is mounted in a flow cell so that short pulses of glucose can be introduced. Assays are repeated to study the reproducibility between assays of the same magnetic preparation as well as between different loading of particles on the electrode tip. To obtain a high reproducibility the magnetic field must be kept constant. Beads are removed by turning off the current and eliminating the electromagnetic field. The buffer flushing the surface of the electrode removes the beads, and new beads can subsequently be introduced (Fig.7).

20.4. Enzyme Immobilization to Tritylagarose

Tritylagarose (29) binds enzymes in a reversible fashion through hydrophobic forces, providing another kind of reversible immobilization. Several enzymes, such as alkaline phosphatase, β-galactosidase, lactate dehydrogenase, and spleen phosphodiesterase, have been immobilized (29).

Under the proper conditions, all enzyme activity is bound to the column. The apparent activity of the immobilized enzyme relative to assays done in free solution is in the range of 80–90%. The enzyme can be eluted in active form (about 95% of the initial activity) with 25% glycerol. The matrix is reusable after the enzyme elution.

20.5. Enzyme Immobilization via Monoclonal Antibodies

Immobilized monoclonal antibodies represent yet another useful matrix for reversible immobilization. Because these antibodies are monospecific and can be chosen so as to correspond to epitopes on enzyme surface and not directed against enzyme active site, the immobilization does not result in significant loss of activity. Solomon et al. (45) describes immobilization of carbodypeptidase A.

Activity assays indicate that the enzyme retains total peptidase as well as esterase activity after immobilization. When stored in PBS at 4 °C, the preparation retains its activity for several months.

20.6. Partition Affinity Ligand Assay (PALA)

Partition in aqueous two-phase systems was utilized to separate bound from free ligand in a binding assay. A prerequisite for success in these efforts is that the molecular entities have different partition behavior when present individually.

The binding of horseradish peroxidase (HRP) to the lectin Concanavalin A (101) serves as a model. The glycosylated enzyme is utilized as an enzyme-

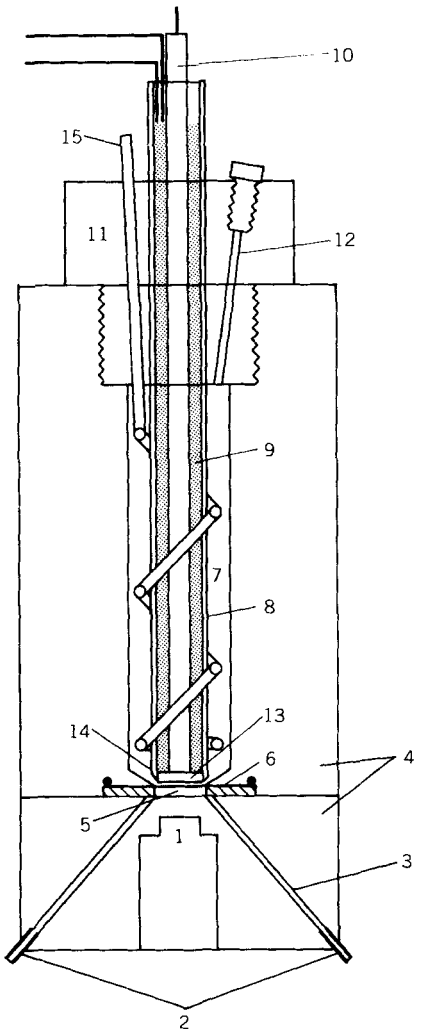

Fig. 7. A flow-through oxygen electrode: 1, water circulation space; 2, stainless steel tubes, 1.2 mm; 3, sample flow tubes, 0.8 mm; 4, Plexiglas block; 5, sample chamber; 6, Teflon membrane; 7, saturated KCl electrolyte; 8, 1-mm-thick glass tube; 9, double-wound solenoid; 10, steel rod, 4 mm diameter; 11, Plexiglas cap; 12, electrolyte supply channel; 13, steel disk, 8 mm diameter, 1 mm thick; 14, 0.5-mm-thick platinum cathode; 15, Ag anode. The magnetic particles carrying the enzyme are introduced through one of the sample flow tubes and the particles get trapped at the surface of the electrode by the electromagnetic field. (From Miyabayashi and Mattiasson (142).)

labeled carbohydrate. Since the interaction studied takes place between the carbohydrate part of the enzyme and the lectin, it was possible to displace the enzyme by additions of free sugars. Utilizing this competitive binding makes it possible to quantify free carbohydrates.

On studying the partition behavior of both native Con A and HRP in a phase system consisting of 13.5% (w/w) PEG-4000 and 13.5% (w/w) $MgSO_4 \cdot 7H_2O$ it was obvious that both proteins favored strongly the salt-rich bottom phase. To design a binding assay, Con A had to be modified to favor the PEG-rich top phase instead. This was achieved by coupling PEG to the molecule.

The modified Con A is partitioned in the phase system. To determine the partition constant accurately, ^{125}I-labeled, PEG-modified lectin is used. The parti-

tion constant (concentration in top phase over that in the bottom phase) was 80, as compared to 0.031 for native Con A. This means that the modification changed the partion constant by almost 2600 times.

A fixed amount of the glycoprotein (HRP) and varying amounts of a free carbohydrate with specificity for Con A are mixed. The modified Con A is added. After the binding reaction is complete, partitioning is carried out in the two-phase system. Alternatively, binding, equilibration, and partitioning take place in the two phase system. The latter approach has proven very successful, for example, with digoxin–antidigoxin interactions (100).

20.7. Monitoring Enzymes That Degrade Macromolecular Substrates by Extractive Bioconversion in Aqueous Two-Phase Systems

Aqueous two-phase systems offer a unique possibility to operate with temporarily immobilized enzymes without sterical hindrances, as is often the case with traditionally immobilized preparations. The use of dye-modified macromolecules as substrates for monitoring enzymes degrading macromolecules has been proven successful.

In an initial experiment, dextranase activity was quantified by exposing the enzyme to a solution of blue dextran (Pharmacia Biotechnology, Uppsala, Sweden) (20 mg/mL) in 0.1 M sodium phosphate buffer, pH 6.0. After incubation for suitable time intervals at 37 °C, an aliquot was removed and added to a two-phase system consisting of 30% (w/w) of polyethylene glycol and 30% (w/w) of $MgSO_4 \cdot 7H_2O$. The liberated dye was recovered from the top phase, whereas nondegraded blue dextran remained in the bottom phase. Absorbance in the top phase at 620 nm gave good correlations to the amount of enzyme added (103).

Aqueous two-phase systems are suitable environments for polymer-degrading enzymes (98, 131). In most cases polymer–polymer systems (e.g., 5% polyethylene glycol (w/w) and 10% Reppal PES (w/w) (from Reppe Glycos AB, Växjö, Sweden) are used when operating with enzyme activity in the phase system, since salt results in ionic strengths that are too high and might interfere. Assays of enzymes by exploiting degradation of dyed substrates can therefore be performed in the two-phase system directly.

20.8. Modified Carbon Paste Electrode

Using dehydrogenases in analysis is often hampered because of problems with retention and recovery of the cofactor. Modifying electrodes by mediators and more recently with mixtures of mediators and cofactors opens new avenues in bioanalysis as discussed by Miki et al. (88).

A mediator or a mediator and NAD^+ are mixed in an electrode prepared from carbon paste and liquid paraffin (132). The adsorption of mediators is a quick process (10 s to 2 min) (87). When designing a system for glucose-6-phosphate, using an electrode with a tip surface of 0.09 cm^2, glucose-6-phosphate dehydrogenase and diaphorase are added to the electrode tip already supplied with

the mediator 2-methyl-1,4-naphtoquinone and NAD^+ (6.54%). To keep the reagents in place, the electrode tip is covered by a dialysis membrane, and to improve stability a nylon net is placed outside the dialysis membrane. The electrode is stored in phosphate buffer, pH 7.0, at 5°C. Measurements are carried out in deoxygenated buffer 0.1 M, pH 8.5.

20.9. Immobilization of α-Chymotrypsin on Celite for Use in Organic Solvents

Chymotrypsin from bovine pancreas is dissolved (31.8 mg/mL) in 50 mM sodium phosphate buffer pH 7.8, and immobilized by drying onto the support. Fifty microliters of enzyme solution is mixed with 50 mg of celite. The preparation is dried under vacuum overnight. The immobilized enzyme preparation is added to an organic solvent (like hexane, chloroform, or toluene). Substrates are dissolved in the organic phase. Water must be added to obtain enzyme activity, but the amount of water present must be carefully controlled.

The enzyme stays on the support, because it cannot dissolve in the organic solvent. However, if more polar solvents are used or increasing amounts of water are added one may encounter leakage of enzyme from the support (121). In such cases, covalent immobilization must be used.

21. CONCLUDING REMARKS

The evolution of modern bioanalysis has, to a large extent, been coupled to the development of immobilization technology. Most immunochemical binding assays as well as all biosensors are dependent on the use of immobilized reagents. Some major challenges for the future include the fabrication of biosensors based on microchips and the validation of biosensor technology in routine applications.

The time has come for the sensors to be taken from the well-controlled environment of the research laboratory and applied to real conditions. This means that the sensors must be robust and able to stand rough conditions in terms of, for example, humidity, temperature, and exposure to organic solvents. When applying the sensors in direct contact with biological fluids biocompatibility will be a major problem.

These challenges will lead to a continuation of the efforts to find new ways to immobilize and to stabilize the immobilized proteins, both native proteins and those produced through rDNA technology.

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Fundamentals of Dry Reagent Chemistries: The Role of Enzymes

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1. INTRODUCTION

Techniques in clinical analysis have undergone many advances in the last few decades. The basic needs in clinical chemistry are unambiguous analyte-specific assays that provide both identification of sample components and their concentration levels. The importance of this is self-evident, since most substances analyzed are part of a multicomponent biological fluid. Advances in enzyme and immunochemical assay techniques provide ideal systems for component

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analysis in biological fluids. Serum and, to a lesser extent, urine are the most frequently analyzed fluids. In adapting a procedure for clinical analysis, a premium is placed on its accuracy, specificity, and convenience to the user. With this in mind, great effort has been devoted to developing miniature integrated analytical elements for the quantitative analysis of serum analytes based to a great extent on enzymes as integral device components. These devices integrate several conventional analytical steps into a single element (1, 2), including separation steps as well as several self-contained chemical reactions. The development of integrated dry reagent chemistries can be compared to the evolution of miniature electronic devices where many components used in the past are currently integrated into microscopic circuits.

Dry reagent chemistries open a new era in user convenience. The user needs only apply the sample to the element to initiate an analysis; the analysis is usually rapid, taking only a few minutes. No reagent preparation or analyte separations from sample components are required. By virtue of their stability and discrete formats, these elements allow both low- and high-volume testing to be cost effective. These devices are easy to store, requiring less space than reconstituted conventional wet chemistry reagents, and they are easily disposable after use.

One of the earliest dry reagent elements described made use of a glucose oxidase–peroxidase enzyme coupled chemistry to analyze urine for glucose (3). The results were semiquantitative since the color produced by the element was compared with color block standards to estimate glucose concentrations. With the introduction of instrumentation, quantification became feasible (4). Owing to the physical configuration of these elements, reflectance spectroscopy and, where appropriate, front-face fluorescence are used to monitor chemical and physical events. Although reflectance spectroscopy is a long-standing technique, its popularity as a tool for routine analysis has increased with the advent of dry reagent chemistries. This chapter will attempt to describe the basic features of dry reagent chemistry elements, the multiplicity of scientific disciplines involved in element construction, the use of compartmentalization to segregate incompatible components involved in cascade reactions (enzymes, immunochemical, and organic), and the use of reflectance photometry in monitoring assays.

2. BASIC FEATURES OF DRY REAGENT CHEMISTRIES

Most dry reagent chemistries are designed to be self-contained analytical devices. Each element may have several functional zones that are introduced as single layers or combined into one layer during construction. Regardless of the number of layers present, all dry reagent elements have a support function, a reflectance function, and an analytical function (Fig. 1). Some elements may also contain a sample-spreading function.

The support function is usually a layer of material that serves as the foundation of the dry reagent element. It consists of a transparent, thin, rigid plastic or plastic-like material. The support layer may also be opaque and contain the reflective function.

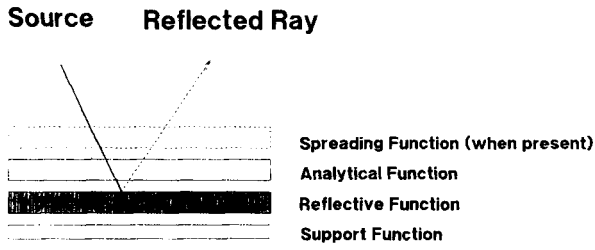


Fig. 1. The basic functions of dry reagent chemistries.

By design, as well as convenience, dry reagent chemistries are monitored by either reflectance photometry or front-face fluorescence. This necessitates the presence of a reflective surface. This surface reflects light emitted or light not absorbed by the chemistry in the element to the detector. A reflective function is usually constructed by introducing reflective (or scattering) centers into a dry reagent element layer. Commonly used materials include pigments (such as TiO_2, $BaSO_4$, MgO, and ZnO), reflective materials (such as metals and foils), and fibrous materials (such as paper and fabrics). The primary requirement of a reflective material is that its absorbance of electromagnetic radiation in the area of interest be negligible.

The analytical function of a dry reagent chemistry is often the most complex. It usually consists of multiple chemical reactions as well as specific physical functions. This may include separation zones, masking zones to hide particular components generated during an analysis, trapping zones to immobilize specific chemical species, and reactive zones where specific chemical reactions take place. These zones may be found as distinct layers in an element or integrated in various combinations into a single layer. Hence, a layer may contain several functions. Layers constituting the analytical function are commonly constructed with film-forming polymers, fibrous materials, or nonfibrous porous materials.

Where present, the sole purpose of a sample spreading function is rapid lateral spreading of a sample after application (5). This feature mediates a uniform sample volume per unit volume of the element (Fig. 2). Substances commonly used to construct spreading functions include fabrics, membranes, and paper.

3. INSTRUMENTATION

Chemical reactions on dry reagent elements are usually monitored by diffuse reflectance photometry (6) and, where appropriate, by front-face fluorescence (7). Since light scattering is common during photometric measurements of dry reagent chemistries, reflectance photometry offers a distinct advantage over transmittance. For reflectance measurements, an analytical element can be illuminated by direct lighting (Fig. 3*a*) or by diffuse lighting. For both direct and

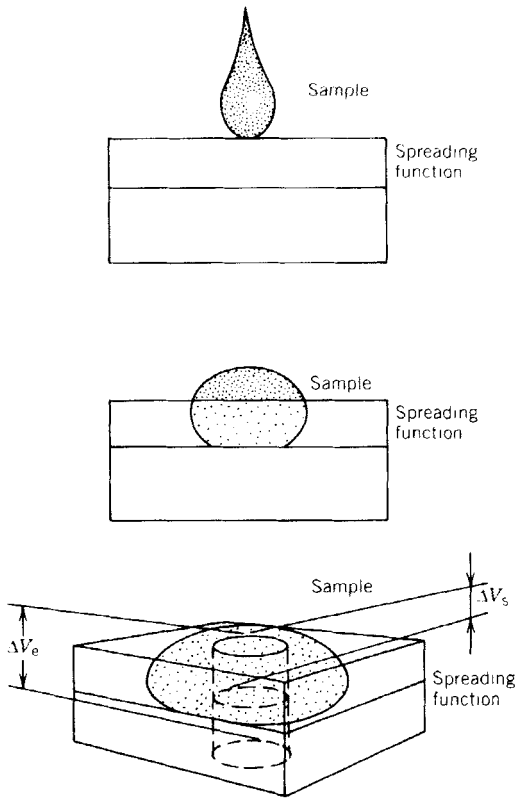


Fig. 2. The spreading function. ΔV_s = unit sample volume, ΔV_e = unit element volume, $\Delta V_s/\Delta V_e$ = constant. (Reprinted, with permission, from *Methods in Enzymology,* Vol. 137. San Diego, CA: Academic, 1988.)

diffuse lighting, two kinds of reflection must be considered. The first is specular reflection, which is mirrorlike reflectance from the illuminated surface where the angle of incidence is equal to the angle of reflectance. This reflection is of limited value in monitoring dry reagent elements. The second is diffuse reflection, which is a reflection from within the reagent layers of the analytical element, and is the predominate reflection mode of interest. Diffuse reflection is not a surface phenomenon, but the result of light interacting with various chemical and physical factors in the layers of the analytical and reflective functions of the element. These factors include absorption, transmission, and scattering properties of the illuminated material.

When the reaction compartment of an element is illuminated at a suitable wavelength, the amount of diffuse light recovered with the aid of the reflective function is a measure of the progress of the reaction, where a chromophore is either generated or degraded. The commonly used expression for reflectance

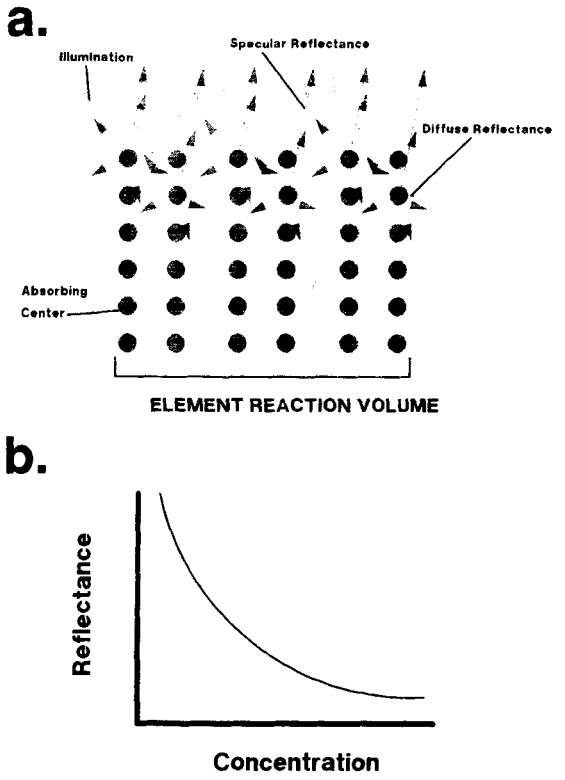


Fig. 3. (*a*) The principle of reflectance photometry. (*b*) The relation of reflectance measurements to concentration.

$$\%R = \frac{I_s}{I_r} \%R_r \qquad [1]$$

describes the amount of diffuse light reflected from the analytical element relative to a known standard, where I_s, I_r, and $\%R_r$ represent, respectively, the reflected light from the sample, the reflected light from the standard, and the percent reflectivity of the standard. Percent reflectance measurements are comparable to transmittance measurements where the relation to concentration is not linear, as illustrated in Fig. 3*b*. To make the reflectance measurements useful and convenient, several algorithms are available to linearize the relation of $\%R$ versus analyte concentration. The two most notable are the Kubelka–Munk (8) and the Williams–Clapper equations (9) (Table 1). The specific algorithm used in an analysis depends on the nature of illumination, the reflectance characteristics of the dry reagent element, and the geometry of the instrument.

TABLE 1

Algorithms That Linearize Reflectance Measurements with Analyte Concentration

Equations	Definitions
Kubelka–Munk	
$C \propto \frac{K}{S} = \frac{(1-R)^2}{2R}$	C = concentration K = absorption coefficient S = scattering coefficient R = % reflectance/100
Williams–Clapper	
$C = \beta(D_T - D_\beta)$	C = concentration β = reciprocal absorptivity
$D_T = -0.194 + 0.469D_R + \frac{0.422}{1 + 1.179e^{3.379D_R}}$	D_T = transmittance density
$D_R = \log R$	D_β = blank density D_R = reflectance density R = reflectance

Front-face fluorescence can be used to monitor fluorescence, either generated or destroyed, within an analytical element (10, 11). The general principle of front-face fluorescence is illustrated in Fig. 4. Irradiating light is passed through a filter, or a light-dispersing device such as a monochromator, to select the appropriate excitation wavelength. With the aid of the reflective function, a portion of the fluorescence and irradiating light is reflected toward the detection system. A filter or monochromator in the detection apparatus segregates the fluorescence from the irradiating light, thus permitting the transmission of fluorescence light only. Unlike reflectance, measured fluorescence is linear with fluorophore concentrations in the absence of self-quenching.

4. CONSTRUCTION OF DRY REAGENT CHEMISTRY ELEMENTS

4.1. Layer Construction

For most purposes, layers of dry reagent elements are constructed by trapping materials in the layer matrix (12). Materials range from small inorganic molecules to large molecular weight enzymes. Although covalent linking of components to a matrix may be necessary in certain cases, the technique is rarely used. Two approaches are most commonly used in constructing element layers. The first involves simultaneous trapping of components and matrix formation and the second depends on trapping of components in preformed matrices.

4.1.1. SIMULTANEOUS COMPONENT ENTRAPMENT AND MATRIX FORMATION

Film casting techniques are commonly used to trap system components and simultaneously form a layer matrix. The technology of thin-film casting has been developed and advanced by industries involved in plastics packaging, electronics, and photographic materials (13–15). The photographic industry has developed techniques for casting thin film layers that contain specific functional chemistries (16, 17). A familiar example is that of color film, which may contain as few as three chemistry layers or as many as 12 in the case of instant color films (18). Each layer serves a specific physical or chemical function in image development and stabilization. Similarly, layers can be constructed in building dry reagent elements. The main differences are that these layers entrap specific chemistries and functions unique and necessary for specific analysis.

A variety of natural and synthetic polymers are used for constructing element layers. The primary requirement is that the polymer form a porous film or be adaptable to membrane formation (19). Gelatin is a natural polymer commonly used in layer construction because of its emulsifying action as well as other chemical and physical attributes (20–22). Enzymes and other reagents can be dissolved or suspended in gelatin emulsions and easily coated onto a support material. Other polymers found useful in layer construction include polyacrylamide (23), polyvinyl alcohol, hydroxypropylmethylcellulose, methylcellulose (24), α-methylglutamate *N*-carbobenzyloxylysine copolymer (25), polyvinyl acetate, agarose, alginate, and carrageenan (26). The choice of polymer for layer construction is a function of porosity needs, compatibility with required chemistries, and any additional analytical functions required in the layer.

One of the crucial parameters that must be controlled during film casting is film thickness. Commercially, this parameter is controlled by coating equipment specifically designed to cast a uniform layer thickness (15). Hence, a variety

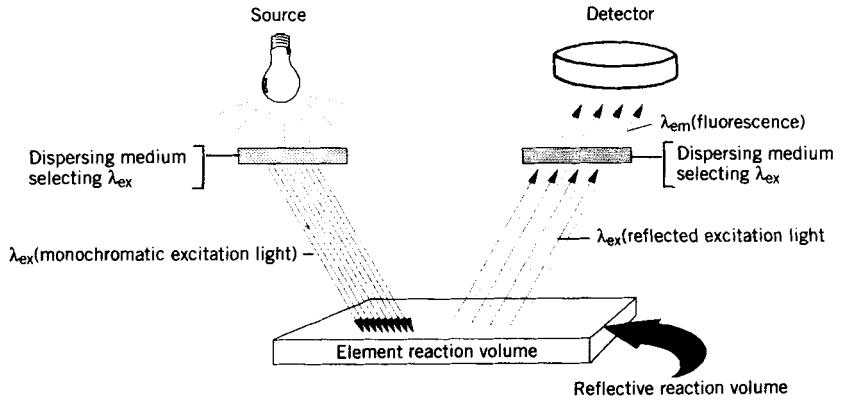
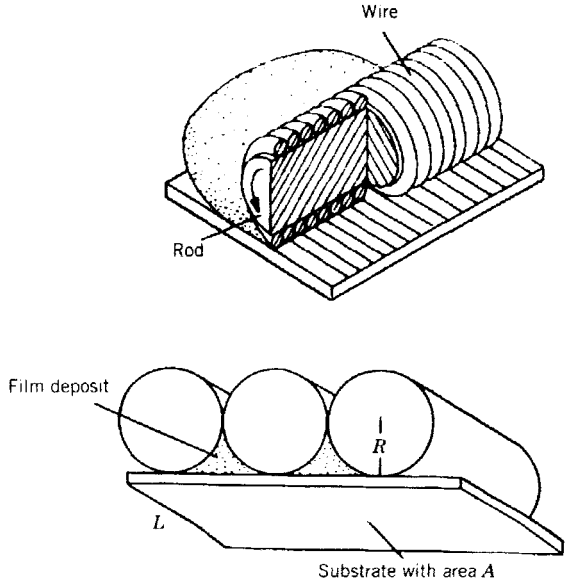


Fig. 4. The principle of front-face fluorescence measurements.

of coating head configurations are available for precision casting of specific materials at desired thickness. However, for rapid, convenient, low-cost coating at the laboratory bench, the Mayer rod technique is most practical (27). This device consists of a cylinder with tightly wrapped wire in a helical configuration, as shown in Fig. 5. The rod is used to pull the coating solution across the surface of a receiving material. The thickness is controlled by the diameter of the wrapped wire. The conversion of Mayer rod wire diameter to expected coating thickness is summarized in Table 2 for several rods.

4.1.2. COMPONENT ENTRAPMENT IN PREFORMED MATRICES

The most widely used alternative approach in layer construction involves trapping components in a preformed matrix by saturation techniques. In this approach, the matrix of choice is saturated with a solution of desired components or reagents. Commonly used preformed matrices include paper, woven fabrics, nonwoven fabrics, and a variety of porous membranes. The availability



$$\text{Unit Volume Deposited} = dV = (1 - \pi/4)2R^2 dL$$

$$\text{Film Thickness Deposited} = dV/dA$$

Fig. 5. Use of the Mayer rod technique in film casting. Unit volume deposited = $dV = (1 - \pi/4)2R^2dl$; film thickness deposited = dV/dA. (Reprinted, with permission, from *Methods in Enzymology*, Vol. 137. San Diego, CA: Academic, 1988.)

TABLE 2

Relation Between Coating Thickness and Wire Diameter of a Mayer Rod

Wire Diameter (μm)	Wet Coating Thickness (μm)
50	5.37
100	10.73
150	16.08
200	21.45
250	26.82
300	32.18
350	37.54
400	42.91
450	48.28
500	53.65

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of reagents during the course of an analysis is controlled by the concentration of reagents in the saturation solution, the thickness and porosity of the matrix, the absorptivity of the solution by matrix material, and the solubility of the reagents in the applied sample.

4.2. Integration of Element Functions

A dry reagent chemistry is designed to simplify what otherwise is a complex multistep analytical procedure for the user. The sole function of the element is to convert an analyte in a sample to a specific quantifiable material. To accomplish this, all physical and chemical functions needed for an analysis must be integrated into one element.

The two basic approaches employed to integrate functions in element construction are shown in Fig. 6. With coating techniques, desired functions as well as incompatible chemistry component can be introduced as separate layers. Hence, the element is constructed by coating a series of layers on top of each other. The alternative approach is to introduce incompatible reagents and functions by a multistep saturation process. By careful choice of solvents, interactions between components can be prevented. The solvents of successive saturations are chosen to prevent dissolution of reagents deposited by previous saturations. This approach allows the construction of single-layer elements that otherwise would require multilayer coating. In some cases, a combination of the two approaches is employed. The final configuration of the dry reagent chemistry element is dependent on the mode of construction and the specific analyte analytical requirements.

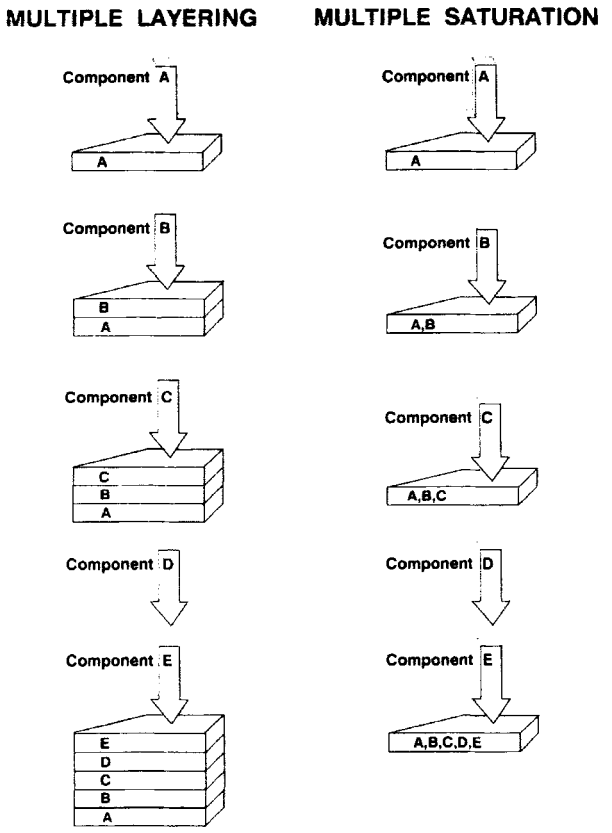


Fig. 6. Comparison of multiple layering and multiple saturation strategies in dry reagent chemistry construction.

5. STABILITY

5.1. Device Stability

Long-term stability in a clinical test device is highly desirable and sought after feature. Of all components, biocatalysts are the most susceptible to degradation in clinical test solutions and are probably the determining factor in the stability of a test device. Many stabilization techniques have been developed for systems and operations that employ biocatalysts as integral system components. Modern biotechnological processes are highly dependent on stabilized biocatalysts. Some of the demands placed on biocatalysts include long-term stability at elevated temperatures, stability under denaturation conditions, and stability to withstand repeated cycles of changing chemical compositions of the media. To meet these demands, advantage has been taken of various advances in enzyme-stabilizing techniques that have been developed over the past two decades. Some

of these include adsorption or immobilization of enzymes to insoluble supports (28), cross-linking of enzymes (29), and encapsulation of enzymes (30). Implementing these techniques has led to easy recovery of biocatalysts in biotechnological processes that often involve complex fermentation and recovery cycles. These developments have helped the industry to implement more efficient and cost-effective operations (31–37).

Although recycling of reagents is not as important in the clinical laboratory as in biotechnology, long-term stability of test chemistries is highly desirable to maintain an efficient and cost-effective operation. Dry reagent chemistries provide the laboratory with a needed stable format for reagents. Due to their discrete nature, the waste resulting from reconstituted and unused reagents is eliminated. Dry reagent chemistries allow the user to store reagents in their most stable state (dry) until use. In the dry state, chemistry components, particularly enzymes, that are otherwise sensitive to destabilizing conditions in an aqueous medium remain stable. In the dry state, destabilizing conditions such as proteolysis and denaturation due to changes in pH, temperature, and ionic strength are prevented. Typically, dry reagent chemistries retain their efficacy for at least two years and often as long as four years.

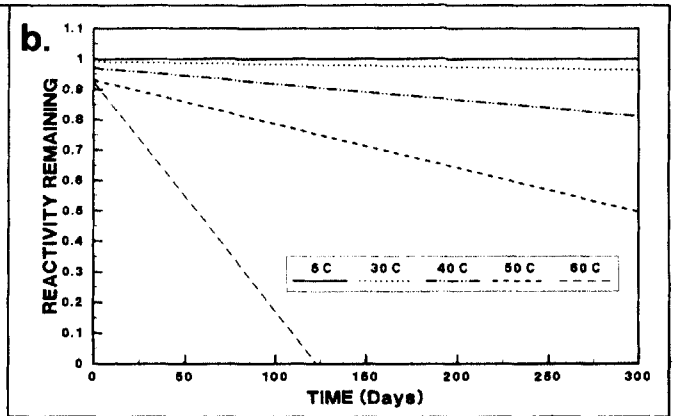
5.2. Estimation of Shelf-Life

Determining the shelf-life of a dry reagent chemistry entails establishing an acceptable storage condition and a storage duration. Requirements at the end of the shelf-life are that the product perform within established specifications. The actual process of determining the shelf-life depends on experimentally mimicking the various environmental stress conditions the test device might encounter during shipping and storage. These may include exposure to varying temperatures, humidity, light intensity, organic vapors, and so on. In practice, temperature is found to be the least controllable parameter. All others can be circumvented by appropriate packaging and only the effectiveness of the packaging configuration needs to be established. The crucial requirements in packaging are provisions of vapor and radiation barriers for the dry reagent chemistry. Examples of packaging that provide such barriers include desiccated foils, amber glass containers with desiccants, and opaque plastic containers with desiccants. Once the packing integrity is established, shelf-life data are derived from thermal degradation studies.

Shelf-life estimation involves developing a thermodynamic model of the dry reagent chemistry. This model is used to make projections of the shelf-life and the estimates are continuously compared with real time data to substantiate the validity of the model or to revise it. A typical study to create a database for shelf-life estimation may consist of subjecting a dry reagent chemistry, in its final packaging format, to continuous thermal stress for 2–3 years in the temperature range of 0–70 °C. An example of a schedule for such a study is shown in Fig. 7*a*. At each check point, dry reagent chemistries are removed from each of the stress conditions, allowed to come to thermal equilibrium, and analyzed, and the per-

a.

DURATION (days)	TEMPERATURE °C							
	0	10	20	30	40	50	60	70
1	X	X	X	X	X	X	X	X
3	X	X	X	X	X	X	X	X
7	X	X	X	X	X	X	X	X
14	X	X	X	X	X	X	X	X
28	X	X	X	X	X	X	X	X
56	X	X	X	X	X	X		
91	X	X	X	X	X	X		
130	X	X	X	X	X	X		
182	X	X	X	X	X	X		
273	X	X	X	X	X	X		
364	X	X	X	X	X	X		
455	X	X	X	X	X	X		
546	X	X	X	X	X			
637	X	X	X	X	X			
728	X	X	X	X	X			



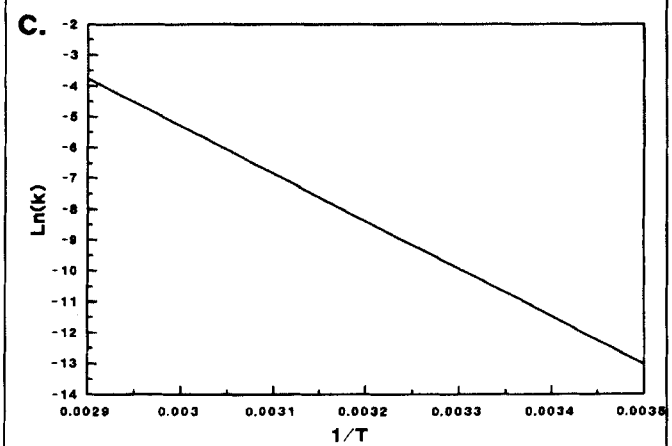


Fig. 7. (*a*) Sample schedule for shelf-life estimation. (*b*) Sample plot of reactivity remaining vs. time after thermal stress. (*c*) Arrhenius analysis of decay rate vs. temperature.

centage of reactivity remaining is recorded. The resulting data are used to construct plots of percent reactivity remaining against duration of stress, similar to the example shown in Fig. 7*b*. The rate of reactivity decay can be estimated from such a plot for each stress temperature. An Arrhenius analysis is then employed (38) to construct a plot of reactivity decay rates against temperature:

$$\ln k = \ln A - E_a/RT \qquad [2]$$

where k is the rate of reactivity decay, A is a constant characteristic of the system, E_a is the activation energy, T is the absolute temperature (kelvins), and R is the universal gas constant. An example of such a plot is shown in Fig. 7*c*.

The experimental relation of $\ln k$ versus $1/T$ provides a means of calculating the amount reactivity anticipated after a given duration of storage at a specific temperature in the range of 0–70°C. This projection scheme allows the manufacturer of the dry reagent chemistry to estimate the allowed storage period for a given temperature and still have the product perform to specification. This information is crucial for the manufacturer in defining the minimum and maximum storage and shipping temperatures as well as the allowed storage duration (shelf-life) of the product in that temperature range. As the database grows, the projections are continuously verified with real-time data.

6. SURVEY OF DRY REAGENT CHEMISTRIES IN CLINICAL ANALYSIS

Dry reagent chemistries have been described for the analysis of a variety of blood constituents. These include metabolites, enzymes, electrolytes, hormones, and therapeutic drugs. A partial list is presented in Table 3. With the exception of electrolytes, nearly all analyses depend on enzyme-mediated chemistries and that includes immunochemical assays. A brief survey of element structures will illustrate how physical functions and chemical reactions used in conventional multistep procedures are integrated in the construction of dry reagent test devices. These examples will illustrate how reactions in dry reagent elements can be compartmentalized and how end products are shunted to other compartments for further reaction. In its final form, each element provides a complete analytical procedure.

6.1. Metabolites

The oldest example of an integrated dry reagent chemistry for quantitative analysis of a metabolite is the Dextrostix reagent strip (Miles Diagnostics) for whole blood glucose analysis. The cross section of the element is illustrated in Fig. 8*a*. The detection chemistry is the well-known glucose oxidase–peroxidase procedure. Approximately 50 μL of whole blood is applied to the surface of the

TABLE 3

Analyses for Which Dry Reagent Chemistries Have Been Reported

Category	Analyte
Metabolites	Bilirubin
	Blood urea (BUN)
	Cholesterol
	Creatinine
	Glucose
	Triglycerides
	Uric acid
Enzymes and proteins	Alanine aminotranferase
	Albumin
	Alkaline phosphotase
	Amylase
	Aspartate aminotransferase
	Creatine kinase
	γ-Glutamyltransferase
	Hemaglobin
	Lactate dehydrogenase
Electrolytes	Ammonia
	Calcium
	Carbon dioxide
	Chloride
	Potassium
	Sodium
Therapeutic drugs	Amaikacin
	Carbamazepine
	Gentamicin
	Phenobarbitol
	Phenytoin
	Primidone
	Quinidine
	Theophylline
	Tobramycin
Hormones	Insulin
	Thyroxine

element (0.5 × 1.0 cm) where plasma glucose is separated from red blood cells by element matrix. After a 1-min reaction time, the red blood cells are removed by washing, and the developed color is monitored from above with a Glucometer reflectance photometer and translated to glucose concentrations. The basic functions of the element are easily identifiable. The element foundation is the support function constructed of a transparent plastic-like material. The analytical function consists of a film membrane cast on top of a paper matrix that contains the detection chemistry. The membrane excludes red blood cells from the detection chemistry and quantitatively meters a plasma volume. The paper matrix also provides the reflective function. The Dextrostix strip has reduced blood

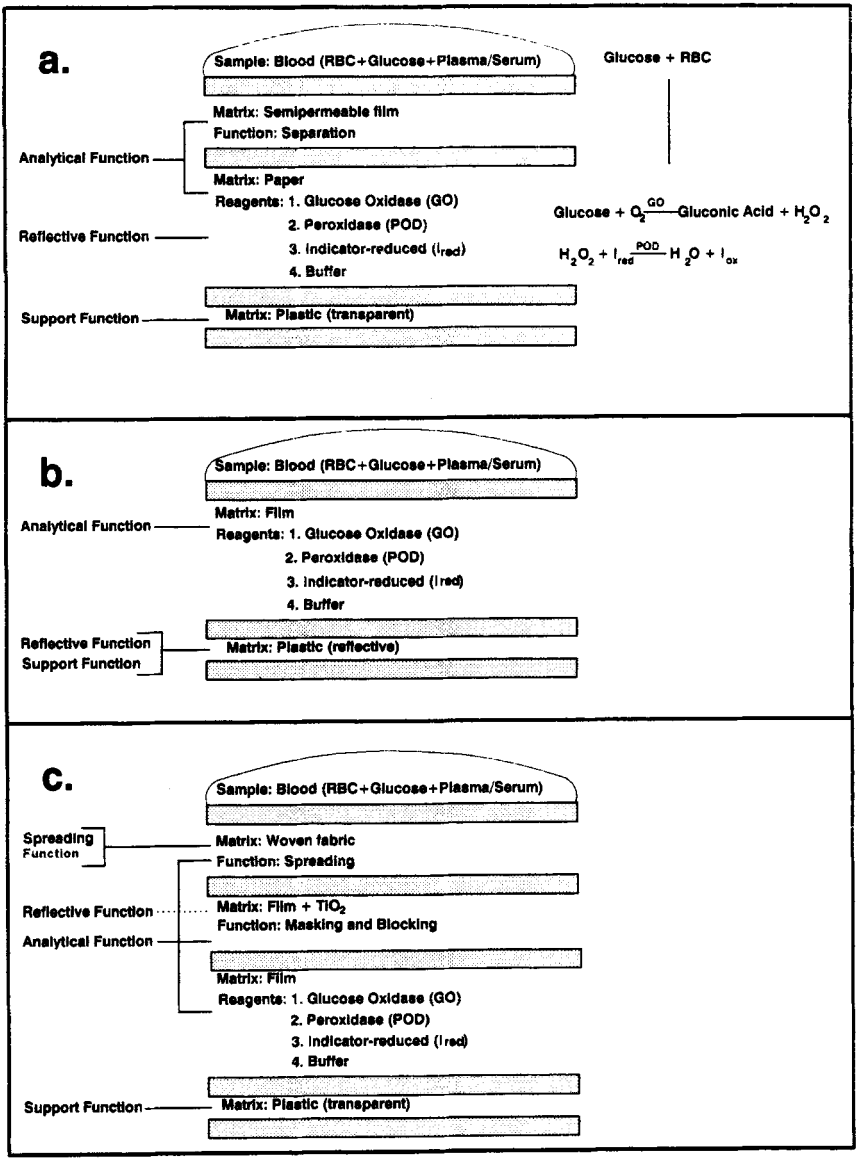


Fig. 8. Cross sections and chemistries of test elements for blood glucose analysis. (*a*) Miles Diagnostics, Inc., (Dextrostix reagent strips), (*b*) BMC, and (*c*) Fuji.

glucose analysis from a multistep laboratory process to a simple procedure that allows diabetics to routinely monitor their blood glucose at home.

Elements with similar functions but different construction and configuration are also available. For illustrative purposes, the cross sections of two elements, developed by Boehringer Mannheim Corporation (BMC) (39) and Fuji Photo

Film Co., Ltd., (40, 41) are illustrated in Fig. 8*b* and *c*, respectively. In the BMC element, the porous film matrix that excludes the red blood cells also contains the detection chemistry. After a specified reaction period, the cells are removed by wiping the surface with an absorbing material (e.g., a cotton ball), and the color produced is analyzed. As with Dextrostix strips, the reflective zone is part of the support layer and the element is monitored from above. Instead of removing red blood cells, the Fuji element makes use of a masking layer, which excludes the cells, serum proteins, and platelets from the reaction layer. The masking layer prevents the optical interference of red blood cells with instrumental measurements. After the sample is spread by the spreading layer, which consists of a woven, thin, fibrous web with an active surface, the glucose diffuses through to the reaction layer, which contains the detection chemistry. The chemistry is monitored from below through the transparent support layer.

The adaptation of small instruments and dry reagent chemistries for home self-monitoring of blood glucose by the diabetic led to the appearance of more complex and easier to use test devices. One example is the Answer system (marketed and manufactured by Cascade Medical, Inc.). The Answer system consists of a pocket-size instrument and a reagent strip. The instrument has several user-convenient features, including a built-in lancing mechanism that accommodates a commercial lancet (Fig. 9*a*). Each strip integrates reagent lot calibration information that is transmitted automatically to the instrument during the test routine. This automatic calibration feature conveniently alleviates the burden of user calibration and prevents potential user errors. The strip, illustrated in Fig. 9*b*, consists of a sample containment plastic housing that contains a reagent membrane with a glucose oxidase–peroxidase chemistry. When a drop of blood is placed in the sample bowl, the wick transports the sample onto the reagent membrane to initiate the analysis. From a user's perspective, the procedure is simple. The user inserts a strip into the instrument, places a finger on top of the sample bowl, releases the lancet, and places the finger puncture drop of blood onto the wick. The user's blood glucose concentration is displayed on the instrument's LCD in 90 s or less.

An element making use of a semipermeable membrane to separate a gaseous product is exemplified by the Ektachem (Eastman Kodak) slide for creatinine analysis (42, 43). A cross section of the element is illustrated in Fig. 10. It consists of a transparent support material, a layer containing the spreading and reflective functions, and an analytical function having two reagent layers separated by a semipermeable membrane. A 10-μL sample of undiluted serum is applied to the reflective-spreading layer to meter a uniform reaction volume. As the sample enters the first layer, the creatinine is converted to ammonia and *N*-methylhydantoin in a reaction catalyzed by the enzyme creatinine iminohydrolase (CI). The semipermeable membrane acts as a barrier to hydroxyl ions, and allows the diffusion of gaseous ammonia into the second reagent layer, where the ammonia *deprotonates* a pH indicator. The color developed in this process is monitored by reflectance from below the carrier. Since the generated dye cannot diffuse across the membrane, the second reagent layer behaves as a trap for immobilizing the end product.

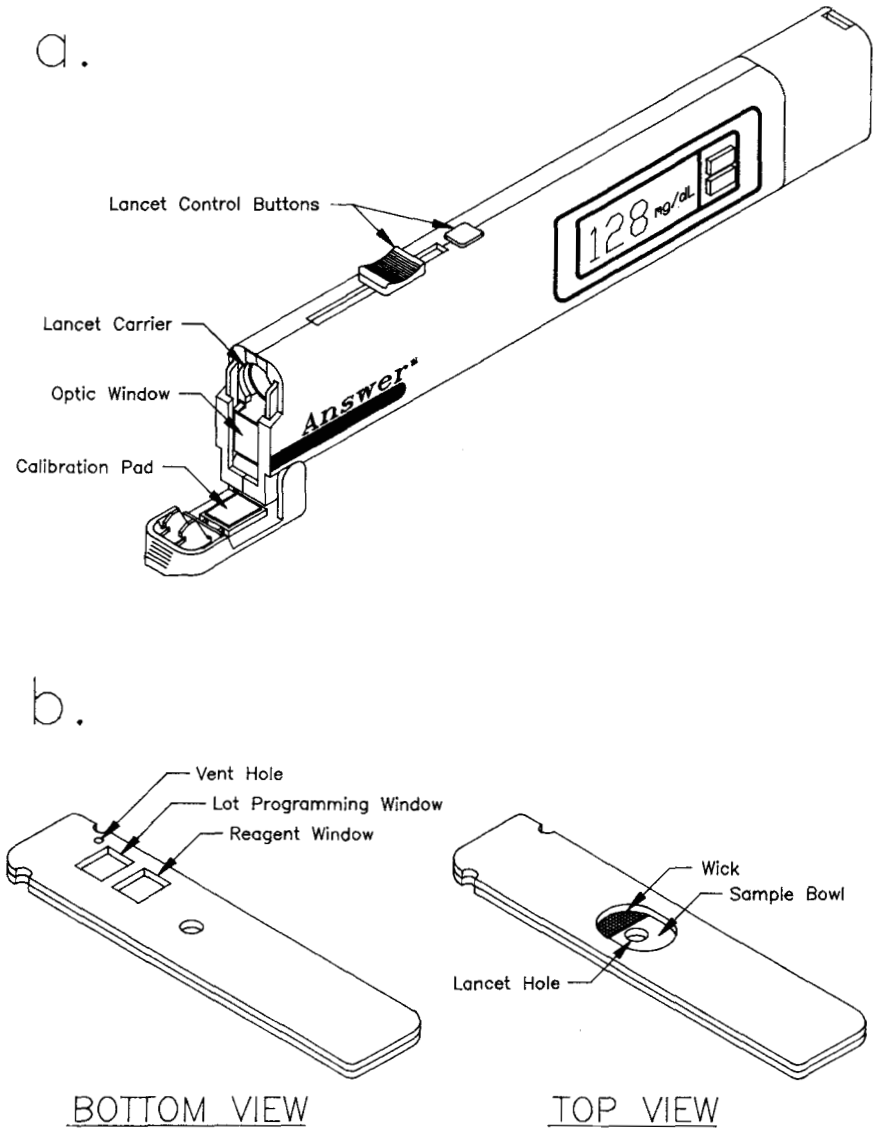


Fig. 9. Features of the Answer (*a*) reflectance photometer and (*b*) reagent strip.

6.2. Enzymes

Constructing dry reagent chemistries for blood enzyme analysis presents new levels of complexity, since enzymes are too large to readily diffuse through most conventional matrices. In addition, many enzyme analyses require coupling multistep reactions which are frequently catalyzed by other enzymes. Some dry reagent matrices have a large, open lattice that allows free diffusion of macro-

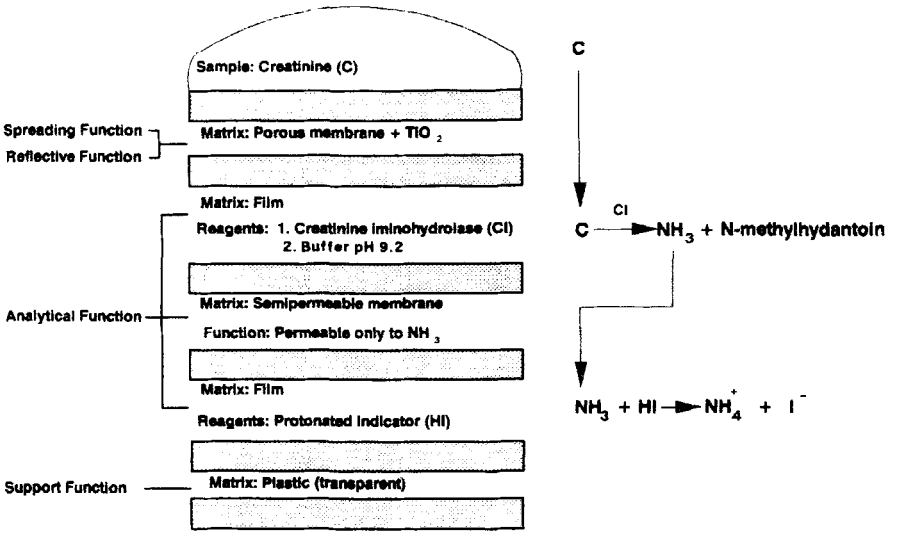


Fig. 10. The cross section and chemistry of Eastman Kodak's Ektachem slide for serum creatinine determination.

molecules, whereas others have a lattice that is impermeable to macromolecules. Both configurations can be used to construct elements for enzyme analysis. An example of an element with an open lattice is the Seralyzer strip for aspartate aminotransferase (AST) (41) (Miles Diagnostics). The cross section of the element is shown in Fig. 11*a*. The element consists of a paper matrix fixed onto a reflective support. On application, the sample spreads into the reagent zone by capillary action. The chemistry couples an oxaloacetate decarboxylase (OAC) reaction, a pyruvate oxidase (POP) reaction, and a peroxidase (POD) reaction to oxaloacetate produced during transamination. The dry reagent strip requires 30 μL of solution derived from a threefold dilution of a serum specimen.

A dry reagent chemistry with a matrix lattice that is impermeable to macromolecules is exemplified by the Ektachem slide(Eastman Kodak) for the analysis of alanine aminotransferase (ALT) (45). A cross section of the element is illustrated in Fig. 11*b*. The format consists of a transparent support layer coated with a reagent layer and a reflective spreading layer. The chemistry is partitioned between these layers. On application of 10 μL of serum, the sample rapidly diffuses into the porous spreading layer. As the sample penetrates the gelatin layer, pyridoxal-5-phosphate (PLP) diffuses into the spreading layer to activate ALT. ALT catalyzes the transamination reaction between L-alanine and α-ketoglutarate. The resulting pyruvate then diffuses into the gelatin layer to oxidize $NADH^+$ to NAD^+. Since the large molecular weight of ALT prevents its diffusion into the gelatin layer, the total chemistry must be mediated by diffusion of low molecular weight substances, such as PLP and pyruvate.

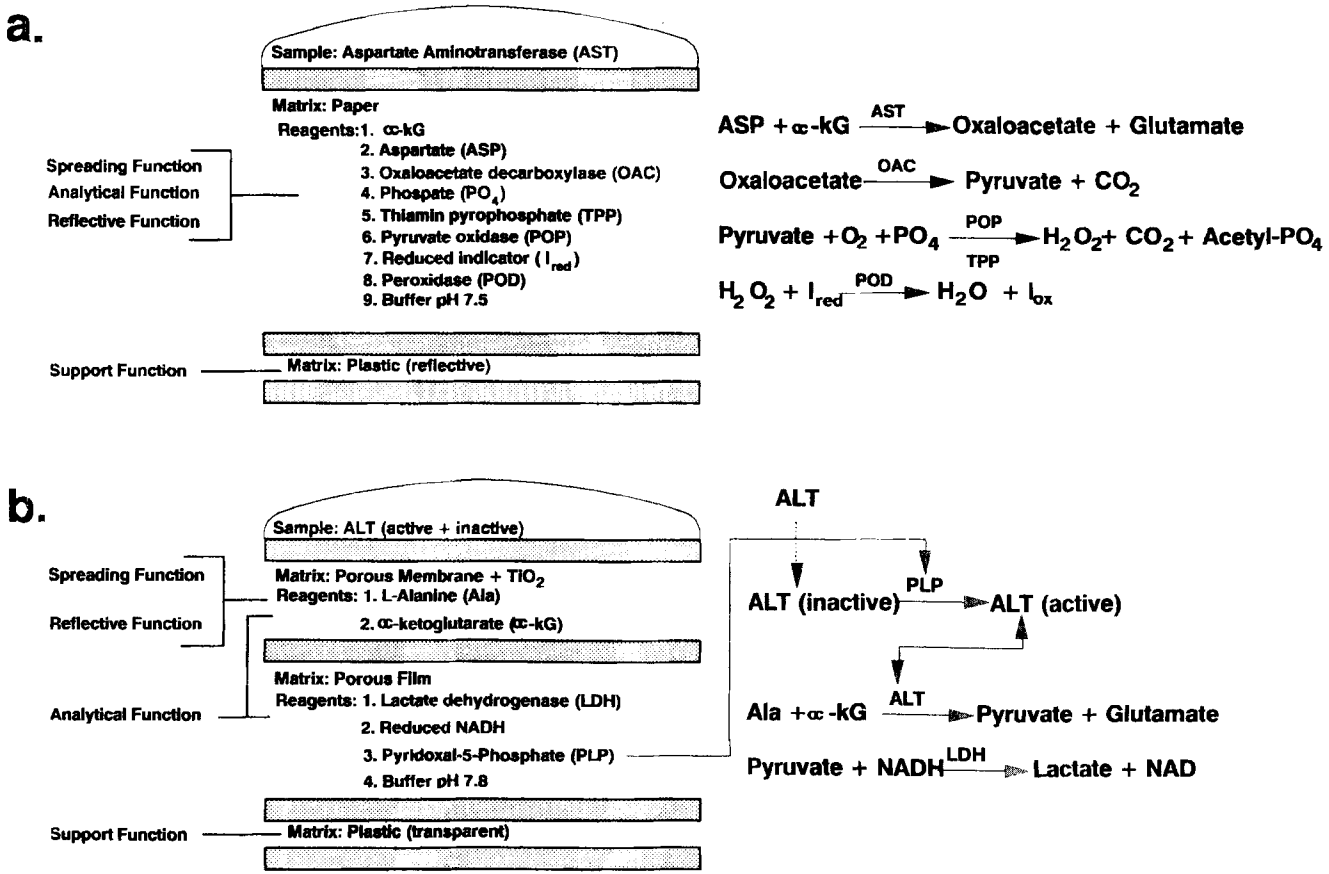


Fig. 11. The cross sections and chemistries of (*a*) Seralyzer strip (Miles Diagnostics, Inc.) for serum AST determination and (*b*) Eastman Kodak's Ektachem slide for serum ALT determination.

A dry reagent chemistry for analysis of an enzyme in whole blood is illustrated in Fig. 12. The Refloquant test element (made by BMC) is used for the analysis of γ-glutamyltransferase (γ-GT) in whole blood (46, 47). The blood sample is applied to the protective mesh, which acts as a spreading layer. The sample then enters a matrix containing glass fibers where the red blood cells are separated from the serum or plasma chromatographically within 15–30 s. The resulting sample continues to diffuse through the bottom paper matrix and comes into contact with the reagent layer. This initiates the chemical reaction, which is monitored through the transparent foil. This device demonstrates the ability of dry reagent devices to separate macromolecules from cells.

6.3. Electrolytes

Dry reagent chemistries are also available for serum electrolyte analysis. Although elements using ion-selective electrochemistries have been described (48, 49), devices based on colorimetric responses are also known (50). The cross section of an element for serum K^+ (Miles Diagnostics) (51) is illustrated in Fig. 13*a*. The element consists of an organic medium containing a potassium-specific ionophore and a dye molecule. On applying a potassium-containing sample, the ionophore mediates a K^+-H^+ exchange between the aqueous and organic phases. On deprotonation, the absorption band of the dye shifts from 460 to 640 nm. The accuracy of the element can be seen in its correlation with gravimetric

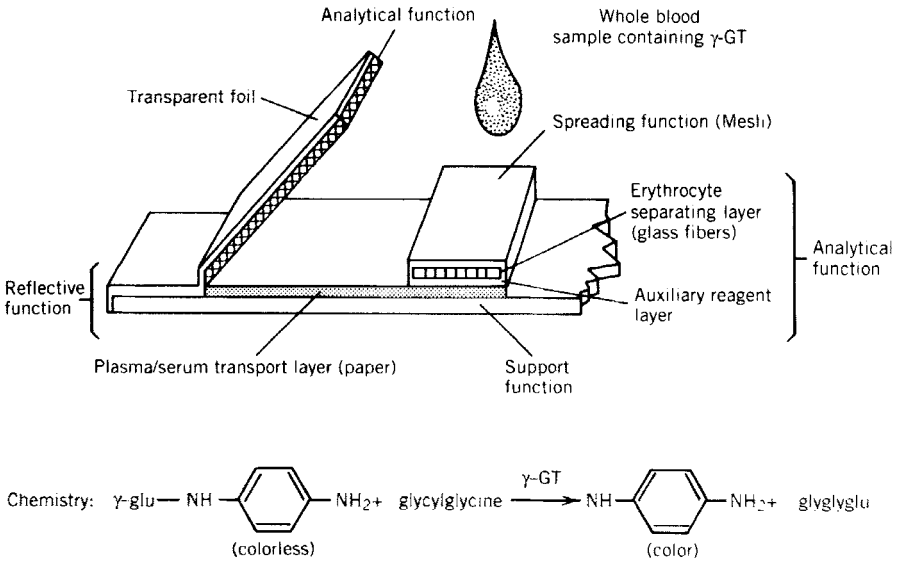


Fig. 12. The cross sections and chemistries of the BMC Refloquant test element for blood γ-GT analysis. (Reprinted, with permission, from *Methods in Enzymology,* Vol. 137. San Diego, CA: Academic, 1988.)

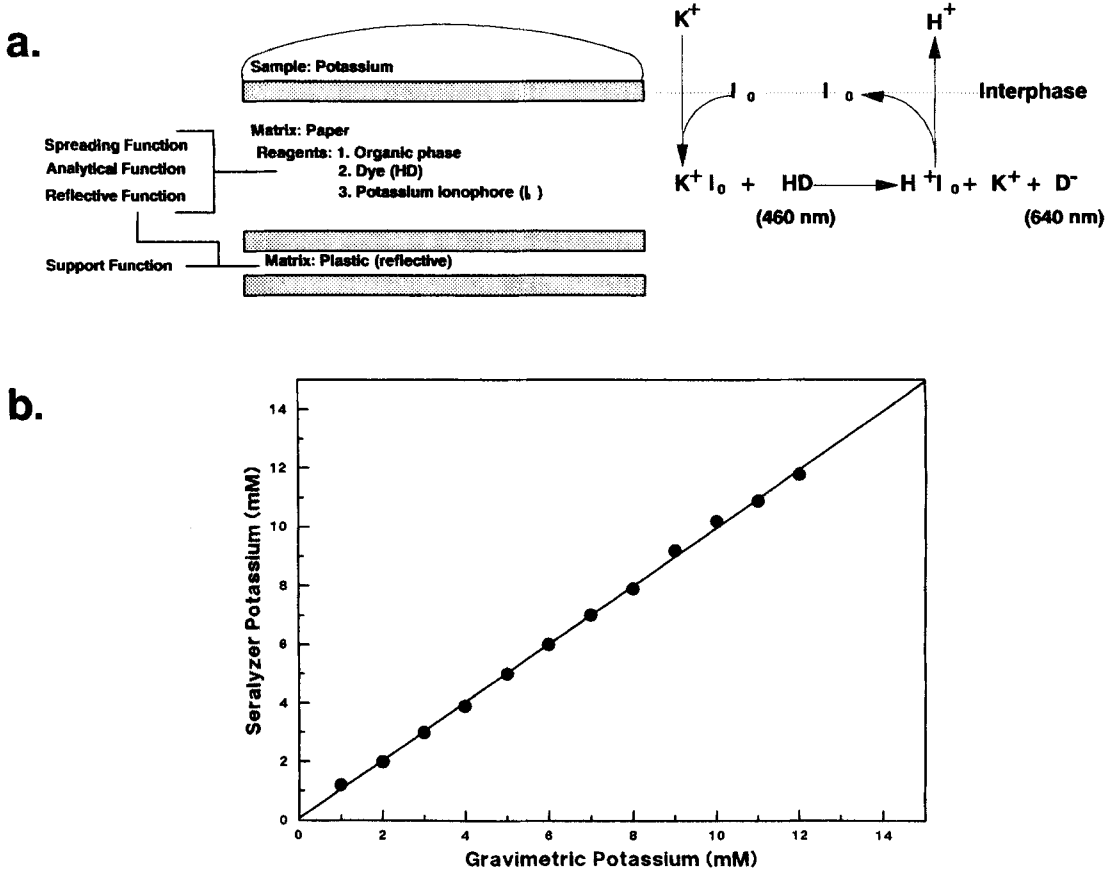


Fig. 13. (*a*) The cross section and chemistry of the Seralyzer reagent strip (Miles Diagnostic, Inc.) for serum potassium determination. (*b*) Comparison of strip and gravimetric analyses for potassium.

methods, as illustrated in Fig. 13*b*. Potassium analyses of serum samples also correlate well with flame photometry (F) (element = 0.988F + 0.092 mM K^+) and ion-selective electrodes (ISE) (element = 0.970ISE + 0.112 mM K^+). This device is a good example of how organic/aqueous partition chemistry may be employed for electrolyte analysis.

6.4. Immunochemical Analysis

Dry reagent chemistries have also been described for immunochemical analyses of therapeutic drugs and hormones. In all cases, the detection chemistry is based on an enzyme assay. Instrumentation and elements for fluorescence immunoassay (11, 52) and for apoenzyme reactivation immunoassay (ARIS) (53) (Miles Diagnostics) for therapeutic drug analysis have been described. The cross section of the ARIS element is shown in Fig. 14*a*. ARIS is a colorimetric immunoassay in which a drug and a drug–FAD conjugate compete for limited number of antibody (Ab) binding sites. Unbound conjugate reactivates apoglucose oxidase. The reconstituted enzyme activity is then proportional to drug concentration in the sample. Figure 14*b* illustrates kinetic profiles of strip color generation as a function of theophylline concentration, and Fig. 14*c* illustrates the proportionality between the rate of color generation and drug concentration.

The cross section of an element described by Fuji Photo Film Co., Ltd. (54) for the detection of the hormone thyroxine is illustrated in Fig. 15. This element is one of the few examples where an assay component is covalently bound to a layer matrix. On applying the sample, the thyroxine (T_4) present in the sample is mixed with the conjugate (thyroxine–peroxidase, POD:T_4). As the solution diffuses through the immobilized antithyroxine antibody (Ab) zone, the free thyroxine and conjugate are partitioned between the matrix-bound antibody and free solution. The free conjugate continues to diffuse through the reflective layer into the detection layer where a glucose oxidase–peroxidase-based assay is initiated. The rate of color development is proportional to the free conjugate concentration, which, in turn, is proportional to the thyroxine concentration in the sample. Elements for immunochemical detection of other analytes have been described by both Eastman Kodak Co. (55) and Fuji Photo Film Co., Ltd. (56).

7. PERFORMANCE

The success of any new technology depends on its correlation to established methodologies. Table 4 summarizes comparisons between dry reagent chemistries made by several manufacturers (44, 46, 56, 57) and conventional solution chemistries. In most cases, the correlation coefficients are greater than 0.95, with the slopes of the correlations close to unity.

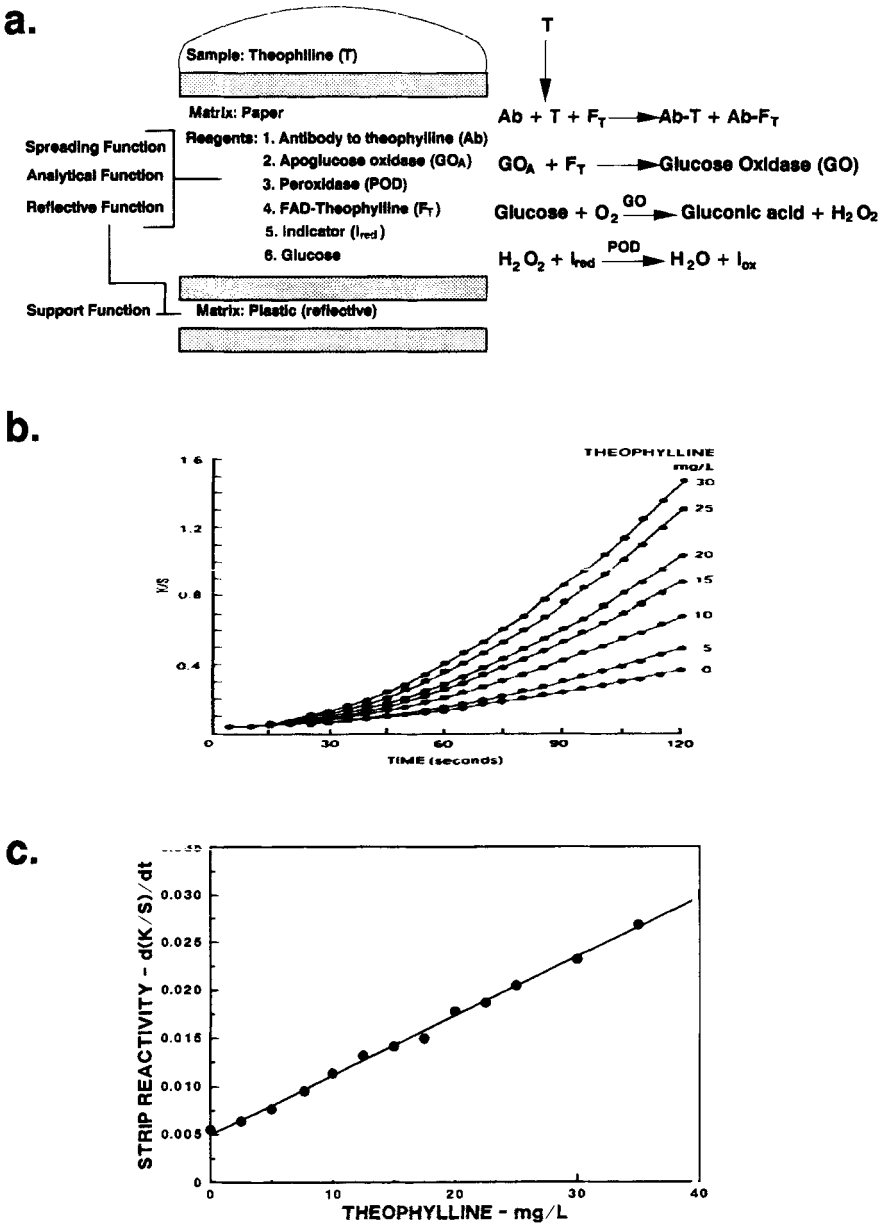


Fig. 14. (*a*) The cross section of the Seralyzer reagent strip (Miles Diagnostics, Inc.) for analysis of serum theophylline. (*b*) The kinetic profile of reacting strip where the change in the reflectance function (*K/S*) is monitored as a function of time for several concentrations of theophylline. (*c*) The relation of theophylline concentration to the rate of change in the reflectance function [$d(K/S)/dt$].

TABLE 4

Comparison of Dry Reagent Chemistries With Reference Methods

System	Manufacturer	Chemistry	Reference Method	N[a]	Regression Equation	R[b]	$S_{y,x}$[c]	Reference
Seralyzer	Ames	ALT	Modified Wroblewski (UV)	74	$y = 0.98x + 1.7$	0.994	5.1 IU/L	(59)
		AST	Modified Karmen (UV)	95	$y = 1.00x + 0.3$	0.991	9.3 IU/L	(44)
		Bilirubin	Jendrassik-Groff	278	$y = 1.04x + 0.1$	0.980	0.33 mg/dL	(56)
		BUN	Berthelot	194	$y = 0.98x + 0.9$	0.950	3.4 mg/dl	(56)
		Cholesterol	Cholesterol-oxidase	114	$y = 1.01x + 3$	0.970	22 mg/dL	d
		Creatine kinase	Rosalki	87	$y = 0.98x + 5.5$	0.998	16 IU/L	d
		Creatinine	Jaffe	162	$y = 1.00x + 0.11$	0.990	0.4 mg/dL	d
		Glucose	Hexokinase	191	$y = 0.99x + 2$	0.990	11 mg/dL	(56)
		LDH	LDH (UV)	154	$y = 1.01x - 4.3$	0.970	28 IU/L	(51)
		Potassium	Flame photometer	80	$y = 0.99x + 0.09$	0.993	0.15 mM	(60)
		Uric acid	Uricase	223	$y = 0.92x + 0.57$	0.980	0.42 mg/dL	(56)
Ektachem	Eastman Kodak	Albumin	SMA II	215	$y = 1.11x - 6.4$	0.81	3.2 g/L	(57)
		ALKP	—	151	$y = 0.96x + 7.1$	—	—	e
		ALT	—	114	$y = 1.01x + 5.2$	—	—	e
		Amylase	ACA	62	$y = 1.20x + 15.4$	0.98	16.7 U/L	(57)
		AST	—	152	$y = 0.99x + 1.7$	—	—	e
		Bilirubin	Jendrassik-Grof	120	$y = 0.97x + 0.03$	—	0.27 mg/dL	f
		BUN	Urease	167	$y = 0.99x - 0.03$	—	1.17 mg/dL	f
		Calcium	SMA II	203	$y = 0.92x + 8.4$	0.90	2.6 mg/L	(57)
		Cholride	C800	180	$y = 1.03x - 4.1$	0.96	1.8 mM	(57)
		Cholesterol	Abell-Kendall	111	$y = 0.98x + 6.3$	—	14.2 mg/dL	f
		CO_2	C800	191	$y = 0.92x + 0.1$	0.76	2.2 mM	(57)
		Creatine Kinase	—	144	$y = 1.11x + 11.3$	—	—	e
		Creatine	HPLC	182	$y = 0.97x + 0.03$	—	0.24 mg/dL	f
		Glucose	SMA II	162	$y = 0.97x - 36.7$	1.000	36.4 mg/L	(57)

TABLE 4 (*continued*)

System	Manufacturer	Chemistry	Reference Method	N[a]	Regression Equation	R[b]	$S_{y,x}$[c]	Reference
Ektachem								
		γ-GT	—	129	$y = 1.04x - 1.2$	—	—	e
		LDH	—	114	$y = 1.01x + 5.2$	—	—	e
		Potassium	C800	177	$y = 1.03x - 0.1$	0.98	0.1 mM	(57)
		Sodium	C800	101	$y = 1.07x - 9.3$	0.91	2.7 mM	(57)
		Triglycerides	CentrifiChem	149	$y = 1.11x + 61.8$	0.98	124 mg/L	(57)
		Uric acid	SMA II	184	$y = 0.98x - 2.4$	0.98	2.4 mg/L	(57)
Reflotron	Boehringer Mannheim	Hemoglobin	Cyanmethemoglobin	200	$y = 1.12x - 0.61$	0.984	—	(46)
		Cholesterol	Cholesterol-oxidase	214	$y = 0.95x + 4.5$	0.977	—	(46)
		γ-GT	Eppendorf	90	$y = 0.96x + 9.8$	0.994	—	(46)
Dry Chem	Fuji	Glucose	Glucoroder-S	124	$y = 0.99x + 3.2$	0.993	8.8 mg/dL	(40)

[a] Number of samples tested.
[b] Correlation coefficient.
[c] Standard error of estimates.
[d] Seralyzer products package inserts, Miles Diagnostics.
[e] *Kodak Ektachem Products News*, Vol. 2, 1983.
[f] Ektachem products package inserts, Eastman Kodak.

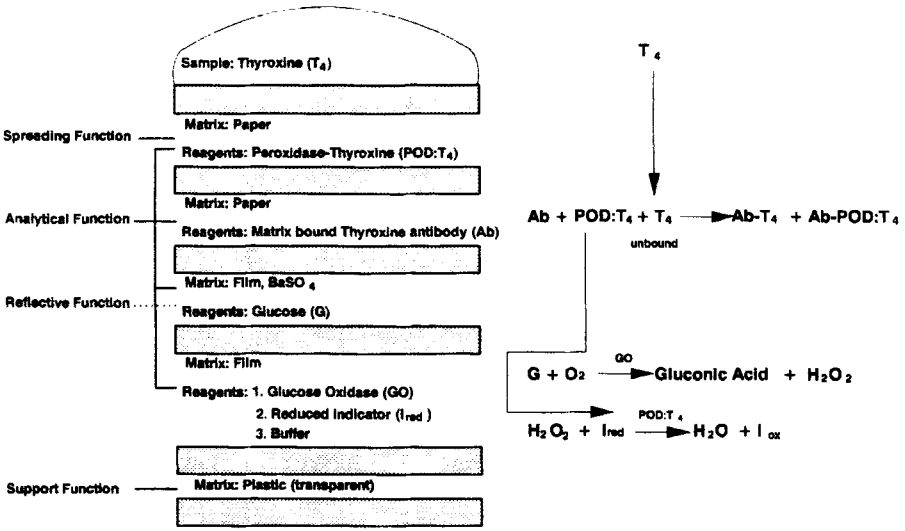


Fig. 15. The cross section and chemistry of a Fuji (Fuji Photo Co., Ltd.) element for thyroxine determination.

8. SUMMARY

It is evident that the specificity and catalytic efficiency of enzymes has revolutionized clinical diagnostics. Developing dry reagent systems provides convenience to the user as well as devices that are more versatile and suitable for a variety of analyses. Most dry reagent chemistries are usually less than 7 $cm^2 \times$ 300 μm thick and are packaged as discrete test devices, which reduces spoilage of unused reagents. Sample volumes needed for analysis are usually in the range of 3–30 μL; the 10-μL volume is most commonly used. The use of such small volumes makes these devices suitable for neonatal and geriatric patients where large sample volumes are not often available. Hence, 150 μL of serum (approximately 300 μL of blood) is sufficient for at least 15 different analyses on a sample. Dry reagent chemistries are easy to store, readily available for use, and disposable. Only application of a sample is needed to start an analysis.

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Enzyme Electrode Biosensors: Theory and Applications

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1. INTRODUCTION

Biosensor technology is entering a very active phase, as is reflected by the vast number of scientific papers, books, reviews, and symposia devoted to sensors (1–6). In the rapidly growing biotechnology and food processing areas, in clinical laboratories, and in pollution control, for example, there is a real and pressing need for analytical devices capable of providing rapid and direct information about the chemical composition of a given sample matrix. The most general of such devices is the chemical sensor, which consists of a physical transducer and a chemical selective layer (1).

The utility of a sensor is determined by its selectivity. Hence, biosensors, which combine the high selectivity of a biological element (such as an enzyme) with a physical transducer, are regarded as ideal analytical tools for use in a wide variety of analytical fields. In the early stages of biosensor technology, enzyme electrodes were developed and applied mostly to clinical analysis of glucose (7, 8). Subsequently, biosensors for a great variety of analytes and applications have been developed (2, 4). Table 1 lists the different biological elements that may be combined with various kinds of transducers, provided that the biological reaction with the substrate can be monitored (Fig. 1).

Four fundamental transduction modes are generally encountered: thermal, mass, electrochemical, and optical. Electrochemical detection techniques have been extensively investigated and applied to biosensor construction (1–8). Thermal and mass sensitive sensors, due to their inherent operating principles, are of general applicability in bioanalysis. Several interesting applications have been reported and considerable progress is expected in these fields (1, 9–11). Within the last five years, a rapid growth in optical fiber and planar waveguide physical sensors has influenced the direction of biosensor research. It appears that mass

TABLE 1

Biosensor Components and Detection Modes

Biological Element	Transducer	Measurement
Enzymes	Solid electrodes	Amperometry
Microorganisms	Ion-selective electrodes	Potentiometry
Whole cells (animal, vegetable)	Gas-sensing electrodes	
	Field-effect transistors	
Antibodies	Photodiode, photomultiplier (+fiber optic)	Optical
Antigens	Thermistor	Calorimetry
	Piezoelectric crystal	Mass change

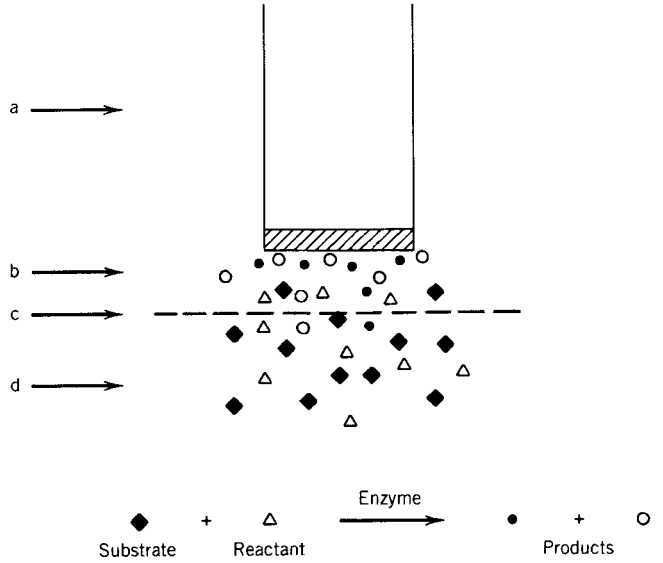


Fig. 1. Schematic view of an enzyme sensor: a, transducer; b, biocatalyst layer; c, permselective membrane; d, solution.

and optical devices will allow a rapid development of immunosensors for medical diagnosis, with possible applications in the area of process control (12–15).

In the field of biosensor technology, immobilized enzyme electrode development occupies a place of prominence due to the attractive performance of this hybrid device. Coupling an immobilized enzyme layer with an electrochemical sensor combines the advantages of using an insolubilized enzyme system (see below) with the sensitivity of readily available potentiometric and amperometric electrodes. The resulting biosensor enables direct, reliable, and reproducible

measurements in many analytical situations, without the need for tedious sample pretreatment or reagent consumption.

A large number of enzyme electrodes have been fabricated and some have been used in commercial applications (see Table 2) resulting in selective and easy to use sensors for a variety of analytes. Enzyme immobilization overcomes the instability, poor precision, restricted linear concentration range, and high cost often encountered with soluble enzymes. The advantages of the immobilized enzyme—reusability, improved stability, lower sensitivity to inhibitors and activators, broader operating range, and economy—when combined with the advantages of specificity and sensitivity provided by the enzyme itself, account for the popularity of the insolubilized biocatalyst as an analytical reagent (16). This chapter describes the history, fundamental principles, fabrication, performance, and applications of enzyme electrodes.

TABLE 2

Commercially Available Biosensors

Manufacturer	Instrument	Characteristics
Analytical Instrument	Glucoroder-E	Immobilized oxidases (GOD) with O_2 electrode
Fuji Electric Co., Japan	Gluco-20 and amylase glucose analyzer	Immobilized GOD, uricase with H_2O_2 detection
Vilnius, USSR	Enzalyst-G	GOD with H_2O_2 detection
Kyoto Daiichi Kagaku, Japan	Glucose Auto and Stat GA-1120	GOD with H_2O_2 detection
Omron Toyoba, Japan	Diagluca	GOD with H_2O_2 detection
Radelkis Electrochem. Instruments, Hungary	Electrode set	GOD with O_2 electrode
Solea-Tacussel, France	Glucoprocessor	GOD and LOD with H_2O_2 electrode
Seres, France	Enzymat	GOD and LOD with H_2O_2 electrode
VEB-MLW Prufgerate-Medingen, GDR	Analyzer ECA	GOD and LOD with H_2O_2 electrode
Yellow Springs Instrument Co., USA	Glucose and YSI industrial analyzer	Immobilized oxidases H_2O_2 detection
MediSense, U.K.	Exactech	GOD and ferrocene
Liston Scientific Co., USA	Eskalab ECS	GOD with H_2O_2 detection
Beckman Instruments, USA	GA2	GOD with O_2 electrode
Universal Sensors, USA	Enzyme electrodes	Various immobilized enzyme electrodes with NH_3, O_2, and H_2O_2 electrodes
Setric G.I., France	Microzym-L	L-Lactate and D-glucose

2. THE CONCEPT OF AN ENZYME ELECTRODE

An enzyme electrode consists of an electrochemical sensor to which a thin layer of enzyme is attached (Fig. 2). Generally, a semipermeable membrane is fixed between the enzyme layer and the solution, between the enzyme layer and the electrode, or both. The resulting probe can operate without pretreatment of the sample since accuracy is achieved independently of the color and turbidity of the solution.

Once exposed to the analyte solution, the substrate penetrates the interior of the enzyme layer. Most enzyme reactions generate or consume an electrode measurable species. As a consequence of the enzymatic reaction, the concentration of both the substrate and the product will change, and after a given period of time a steady state is reached where the rate of product supply and rate of consumption of substrate will be equal. The concentration of the electroactive species is monitored potentiometrically or amperometrically and the electrochemical signal can be correlated back to the concentration of the substrate to be measured. The response of an enzyme electrode can be monitored by a steady-state (i.e., equilibrium) method measuring millivolts, or change of the electrochemical signal can be monitored as a function of time. The construction of enzyme electrode probes demonstrating optimal performances for analytical purposes requires the judicious selection of both the biocatalytic element and the appropriate electrochemical sensor.

2.1. The Biocatalytic Element

Enzyme-catalyzed reactions have been used for analytical purposes for many years in the determination of substrates, activators, inhibitors, and enzymes.

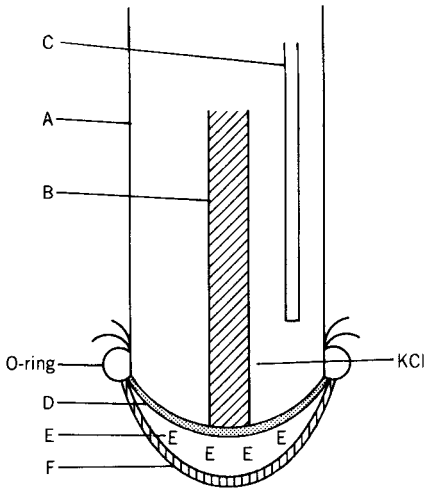


Fig. 2. Schematic structure of a Clark-type enzyme electrode: A, electrode body; B, working electrode (Pt); C, reference electrode (Ag/AgCl); D, inner gas-permselective membrane (polypropylene); E, enzyme layer; F, outer membrane.

Today, more than 2000 enzymes are known. Several hundred have been isolated, many in pure or crystalline form. Over 400 are available commercially, some of them from as many as 10 different sources (8). Because of the relatively recent availability of increasing numbers of highly purified, very active enzyme preparations at reasonable cost, these biocatalysts are now commonly used as laboratory reagents for routine analytical work (16).

Glucose oxidase (GOD) was the first enzyme combined with an electrode and until now has been the enzyme most often applied in practical analysis or in biosensor development. Glucose oxidase is a flavin adenine dinucleotide (FAD) containing enzyme, which catalyzes the oxidation of glucose to gluconic acid. The enzyme is readily available, quite stable in solution, and highly selective (see Table 3). The excellent properties of glucose oxidase, along with the high clinical demand for glucose analysis, account for the vast amount of effort devoted to the development of glucose sensors. Of equal interest, from an electrochemical point of view, is the fact that GOD, like other oxidases, uses the electron acceptor oxygen, consumption of which can be easily monitored by a Clark-type oxygen electrode (see below).

Other oxidoreductases (dehydrogenases) use electron acceptors other than oxygen, such as the cofactor NAD^+ or $NAD(P)^+$. These cofactors may be advantageously combined with electrochemical sensors, provided that the cofactor is externally supplied or immobilized and regenerated. More than 250 highly specific dehydrogenases are commercially available. Hydrolases are another class of readily available enzymes that can be combined with potentiometric sensors. Hydrolases catalyse the hydrolytic cleavage of C–O, C–N, C–C, and other bonds. Other biosensors can be fabricated from the new generation of hybrid and synthetic enzymes (17).

A great number of enzymes have already been combined with electrodes. In some cases, several possible enzymatic routes may be selected, depending on the substrate to be analyzed. One such example is that used to determine aspartame (Fig. 3) (18–20). Enzyme electrodes use, ideally, only one enzyme and monitor the main substrate–enzyme reaction, for example, glucose oxidase–glucose. If the main substrate–enzyme reaction is not electrochemically detectable or if signal amplification is required, bi- or trienzymatic sequences may be applied,

TABLE 3

Specificity of the Enzyme Glucose Oxidase

Substrate	Relative Rate
β-D-Glucose	100
L-Deoxy-D-glucose	12
Mannose	0.98
α-D-Glucose	0.64
Galactose	0.12
Fructose	0.0
Lactose	0.0

such as sucrose (21, 153). The choice of the appropriate enzyme with respect to purity, availability, and activity is of prime importance in the development of enzyme electrodes.

2.2. The Electrochemical Transducer

The choice of an appropriate electrochemical sensor is governed by several requirements: (1) the nature of the substrate to be determined (ions or redox species); (2) the shape of the final sensor (microelectrodes); (3) the selectivity, sensitivity, and speed of the measurements; and (4) the reliability and stability of the probe. The most frequently used sensors operate under potentiometric or amperometric modes. Amperometric enzyme electrodes, which consume a specific product of the enzymatic reaction, display an expanded linear response

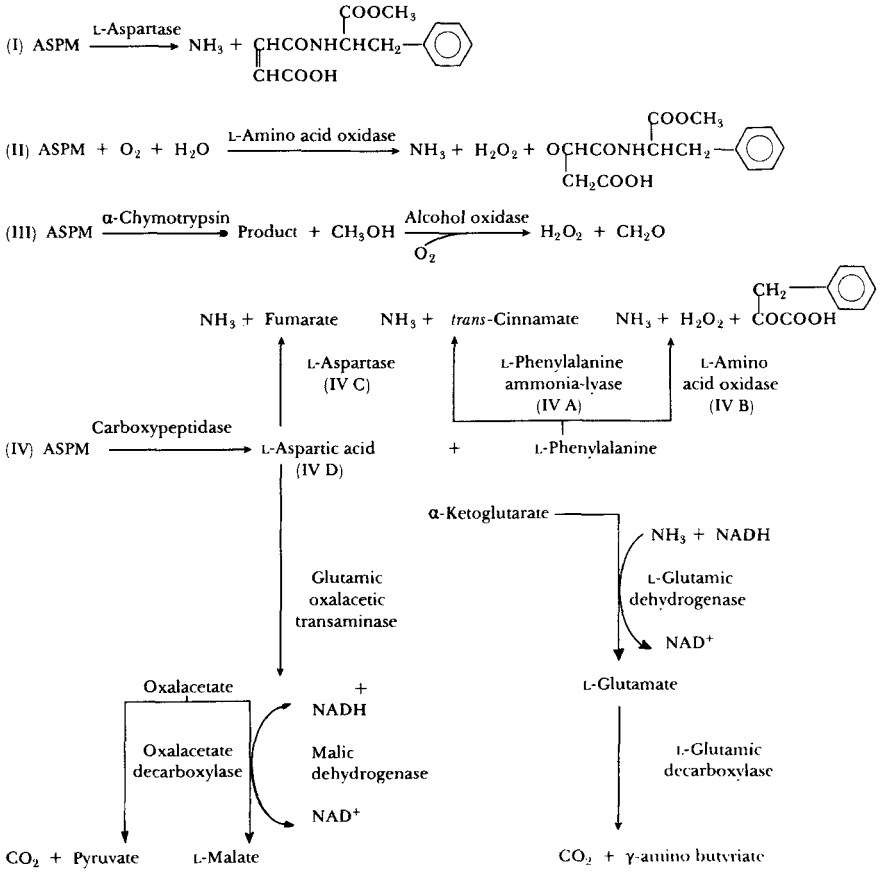


Fig. 3. Possible routes of enzymatic degradation of aspartame.

range and a larger apparent Michaelis-Menten constant (K_m) than the potentiometric enzyme electrodes.

2.2.1. POTENTIOMETRIC SENSORS

In the potentiometric-type sensor, a membrane (glass, solid state, liquid) selectively extracts a charged species into the membrane phase, generating a potential difference between the internal filling solution and the sample solution (enzyme layer). This potential is proportional to the logarithm of the analyte concentration (activity) following the well-known Nernst equation.

Ion-selective electrodes sensing various ions, such as F^-, I^-, S^{2-}, CN^-, NH_4^+, and H^+, have been successfully employed together with glucose oxidase, peroxidase, urease, catalase, and so on (8). However, in using iodide sensors to measure glucose, several types of interferences may be encountered: (1) interference at the iodide electrode (SCN^-, S^{2-}, CN^-, Ag^+), and (2) interference from oxidizable compounds present in blood (uric acid, tyrosine, ascorbic acid, iron(II)), which alter the equilibrium in the oxidation of iodide to iodine (22). The pH and fluoride sensors are the most sensitive and selective commercially available ion-selective electrodes (23) that have been used in the construction of biosensors.

Among the potentiometric ion-selective electrodes, the gas-sensing probes are particularly useful (24, 25). Outstanding selectivity can be achieved simply by casting the ion-selective electrode with a gas perm-selective membrane (polypropylene, Teflon). Potentiometric ammonia- and carbon dioxide-selective gas-sensing devices are the most frequently used because of their selectivity. Figure 4 illustrates a typical biocatalytic potentiometric gas-sensing probe. The electrode consists of a combination of pH glass and reference electrodes and a gas-permeable membrane. Between the glass electrode and the membrane is a thin layer of sodium bicarbonate solution (carbon dioxide sensor) or ammonium chloride (ammonia sensor). When the whole electrode is exposed to a solution of substrate to be analyzed, the enzymatic reaction produces gas (CO_2, NH_3). As the gas diffuses both back to the sample and toward the pH sensor, it passes through the enzyme and the gas-permeable membrane, then dissolves in the internal filling solution, thereby changing its pH and giving rise to the potentiometric response of the system. Linear responses are generally observed in the range of substrate concentration from 10 μM to 0.1 M, with a response time ranging from 1 to 10 min. Unfortunately, the baseline recovery time is of the same order of magnitude, which detracts from the usefulness of this type of sensor. Interference can result only from dissolved species in the sample that can both diffuse through the synthetic membrane and produce a pH change in the thin layer of the internal filling solution.

Other recently developed probes, which consist of potentiometric sensors with solid-state internal contact (1, 26, 27), are expected to bring a major contribution in the biosensor area due to their attractive shape (microsized, no internal solution). But there are some drawbacks related to reproducibility of enzyme immobilization and light sensitivity (28).

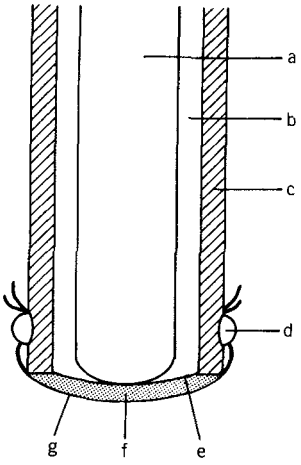


Fig. 4. Cross-sectional view of a potentiometric gas-sensing enzyme electrode: a, glass pH electrode; b, internal filling solution; c, electrode outer jacket; d, O-ring; e, gas-selective membrane; f, enzyme layer; g, dialysis membrane.

2.2.2. AMPEROMETRIC SENSORS

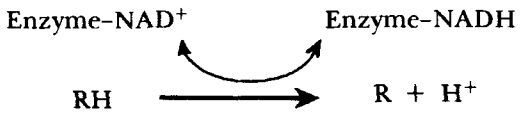
Amperometric sensors monitor current flow, at a selected, fixed potential, between the working electrode and the reference electrode. In amperometric biosensors, the two-electrode configuration is often employed. However, when operating in media of poor conductivity (hydroalcoholic solutions, organic solvents), a three-electrode system is best (29). The amperometric sensor exhibits a linear response versus the concentration of the substrate. In these enzyme electrodes, either the reactant or the product of the enzymatic reaction must be electroactive (oxidizable or reducible) at the electrode surface. Optimization of amperometric sensors, with regard to stability, low background currents, and fast electron-transfer kinetics, constitutes a complete task.

Due to their catalytic response in measurement of hydrogen peroxide oxidation and oxygen reduction, platinum electrodes are usually used as the base sensor in combination with biocatalysts. Also popular are the new carbon substrates, modified or unmodified (glassy carbon, carbon composites), which add new dimensions to the research on amperometric enzyme electrodes (30). Modifying the electrode surface structure by appropriate chemical reagents and hoping that the electrode will take on the properties of the attached reagent is another active area of research in amperometric sensors. Indeed, it is the progress in modified electrode technology that has resulted in (1) acceleration of the electrochemical reaction rate, (2) prevention of fouling electrode surfaces and interference in the electrode reaction, and (3) better control over the enzyme immobilization step.

Redox mediators have been introduced into enzyme electrodes to circumvent the problems associated with the sluggish redox behavior of protein structures at the electrode surface. Indeed, many enzymes involved in the oxidation and reduction reactions contain electroactive centers (hemin, flavin) surrounded by a protein matrix, preventing efficient electron transfer to electrodes.

This may be illustrated by two examples: the dehydrogenase- and oxidase-based electrodes.

Consider the NAD^+-dependent enzyme dehydrogenase, which catalyzes the oxidation of a substrate, RH:



[1]

Ideally, the reaction should be followed by direct oxidation of the reduced NADH-dependent enzyme at the electrode surface. The coenzyme is loosely bound, but is so well embedded in the enzymatic structure that direct electron transfer between the redox center and the electrode requires high overvoltage to overcome the reaction barrier or steric hindrance exhibited by the protein. Moreover, the oxidation of NADH at the electrode may be accompanied by non-specific side reactions, including some NAD^+ decomposition. In addition, the reaction products often cause severe electrode surface fouling which causes supplementary slow heterogeneous electron transfer. Therefore, several mediated electrochemical devices, which contain compounds such as phenoxazine (31, 32) that catalyze the regeneration of the soluble nicotinamide coenzyme of dehydrogenases, have been developed.

Similar surface problems are encountered with the flavoprotein oxidases. The classical glucose sensor is straightforward. The reaction may be divided in the following steps:

$$\text{Immobilized layer: Glucose} + \text{FAD} \xrightarrow{\text{GOD}} \text{Gluconolactone} + \text{FADH}_2 \quad [2]$$

$$\text{FADH}_2 + \text{O}_2 \rightleftharpoons \text{FAD} + \text{H}_2\text{O}_2 \quad [3]$$

$$\text{Electrode: } \text{H}_2\text{O}_2 \rightleftharpoons \text{O}_2 + 2\text{H}^+ + 2e^- \quad [4]$$

Oxidation of glucose occurs enzymatically and the flavin within glucose oxidase is reduced. In turn, oxygen regenerates the enzyme in its oxidized state, while oxygen is reduced to hydrogen peroxide. Two FAD molecules of glucose oxidase are embedded and tightly bound in the protein matrix of a molecular weight of approximately 150000. The binding prevents a direct electrical communication between the redox centers and the electrode surface. Using a mediator-modified electrode to replace oygen as the electron acceptor for GOD offers a potential solution to this problem. Oxygen is no longer required and hydrogen peroxide is not produced; electrochemistry occurs at the redox potential of the mediator, which can be chosen to minimize interference from other electroactive species. To be efficient, a mediator should meet several requirements: It should (1) be insensitive to pH and oxygen, (2) effect fast interaction with the enzyme site and fast electrochemical behavior, (3) be amenable to immobilization. Soluble mediators, such as ferricyanide and benzoquinone, have been applied, but the

relatively water-insoluble ferrocene (bis(*n*-5-cyclopentadienyl)iron and its derivatives have already been successfully applied in several enzyme electrodes suitable for real sample analysis (33–36, 45). Other compounds, such as tetrathiafulvalene (TTF) and tetracyanoquinodimethane (TCNQ), are useful mediators for incorporation in enzyme electrodes and immunosensors (37, 38).

3. PRINCIPLE AND EVOLUTION OF ENZYME ELECTRODES

The concept of combining the enzyme with an electrode was established in 1962 by Clark and Lyons (39) and the first construction of an enzyme electrode was reported by Updike and Hicks (40) for the determination of glucose in biological solutions and tissues. Since then, a number of enzyme electrodes have been constructed and commercialized with varying success. The general trends in this expanding field are illustrated by considering the two major biosensors developed thus far: the amperometric glucose electrode and the potentiometric urea electrode.

3.1. Amperometric Glucose Electrode

In amperometric sensors, the rate of the enzyme reaction is monitored by directly recording the current (electron transfer) that occurs at the electrode interface. Ideally, the catalytic redox behavior of the prosthetic group (or cofactor) of the enzyme is directly monitored at the electrode surface. As previously mentioned, however, the active redox center is generally surrounded by a thick insulating protein layer which prevents direct electron transfer between the enzyme centers and the bare electrode surface. Basically, three distinct classes of amperometric enzyme electrodes may be recognized, depending on the species monitored during the glucose degradation by the enzyme glucose oxidase (see equations 2–4 and Fig. 5).

3.1.1. FIRST-GENERATION GLUCOSE ELECTRODES

The so-called first generation of glucose electrodes relies on monitoring either the consumption of oxygen or the formation of hydrogen peroxide:

$$\text{Glucose} + O_2 \xrightarrow{\text{GOD}} \text{Gluconolactone} + H_2O_2 \qquad [5]$$

In the pioneer work of Updike and Hicks (40), the enzyme glucose oxidase (GOD) was maintained in a layer of acrylamide gel over a "polarographic" oxygen electrode (actually the Clark oxygen electrode). The current output of this probe results from the reduction of oxygen at a platinum electrode and thus is a function of the oxygen tension. When oxygen is not rate limiting and the glucose concentration is below the apparent K_m for the immobilized GOD, there is a linear relationship between glucose concentration and the decrease in

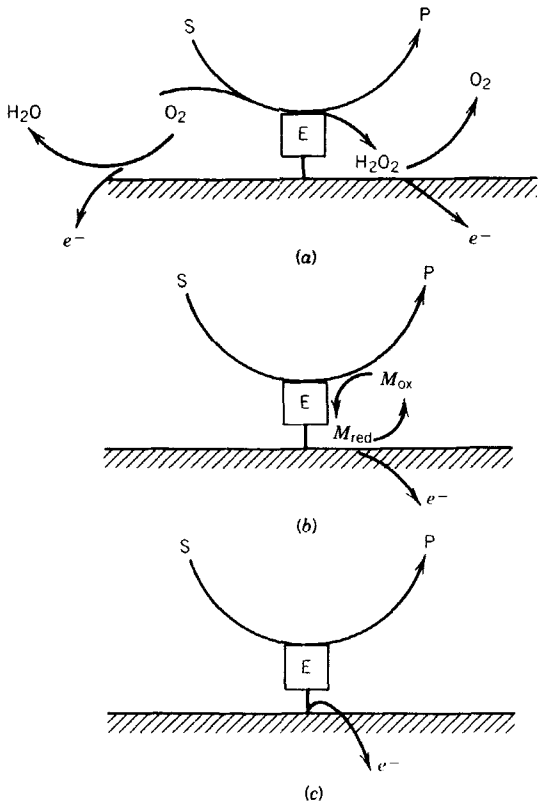


Fig. 5. Schematic illustration of the three generations of glucose electrodes: (*A*) first generation, (*B*) second generation, (*C*) third generation. E, enzyme.

oxygen tension, that is, the decrease in current. This electrode has been appropriately designed to limit perturbation due to changes in flow rate (use of micro-sized platinum cathode) and due to oxygen fluctuation (by using a dual configuration, one cathode covered with active enzyme, the other with inactive enzyme) with differential readout.

The first generation of glucose electrodes used biosensors that monitored the product of the enzymatic reaction, H_2O_2 (41). This is achieved by reversing the polarity of the working electrode and recording the H_2O_2 oxidation current at a fixed potential, for example, +600 mV versus Ag/AgCl. This strategy circumvents the problems related to fluctuation of oxygen tension and allows better sensitivities. Today, most of the commercially available glucose analyzers that utilize immobilized enzyme electrodes still monitor either oxygen consumption or H_2O_2 formation (Tables 2 and 5). Critical evaluations of the performance of these instruments in real sample analysis have been reported (42–44).

3.1.2. SECOND-GENERATION GLUCOSE ELECTRODES

Second-generation electrodes have biosensor configurations that use an electron acceptor (redox mediator), which is able to shuttle electrons from the redox center of the enzyme to the surface of the working electrode:

In the enzyme layer $$\text{GOD-FADH}_2 + M_{ox} \rightleftharpoons \text{GOD-FAD} + M_{red} + 2H^+ \quad [6]$$

At the electrode $$M_{red} \rightleftharpoons M_{ox} \quad [7]$$

The rate of formation of the reduced form of the mediator (M_{red}) is monitored amperometrically (45). During the catalytic reaction, the mediator first reacts with the reduced enzyme, and then diffuses out of the enzyme layer to the electrode surface where it undergoes rapid charge transfer. The early applications of mediated enzyme sensors used a soluble redox mediator, such as hexacyanoferrate(III). Further progress was realized by constructing reagentless sensors, for example, by retaining the mediator physically, electrostatically, or chemically onto or into the electrode matrix. With this respect, electrodes based on organic metals constitute unique configurations (46, 47). Indeed, these electrodes contain in their structure the mediator itself and the latter is continuously supplied at the electrode interface due to slow dissolution of the organic metal (48). Such electrodes result from the formation of stable charge-transfer complexes that is, complexes where a partial transfer of an electron occurs from a donor to an acceptor. A typical complex contains the donor *N*-methylphenazinium (NMP^+) and the acceptor tetracyanoquinodimethane ($TCNQ^-$) (see Table 4). These conducting salts are metallic at room temperature and may be easily machined into various shapes. Alternatively, other electrode material has been employed for a continuous supply of the mediator, for example, benzoquinone in carbon paste (49).

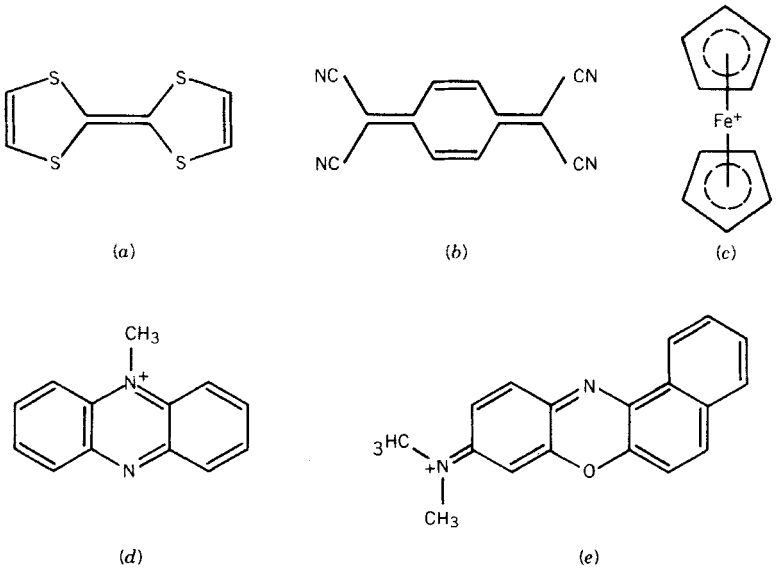
High molecular weight hydrophobic molecules are used to alleviate the leaching of the mediator out of the sensor into the solution (50, 51). Further improvement was obtained by chemically attaching the mediator to the electrode surface (52) to a polymeric network on the electrode (53) or by direct attachment to the enzyme (54, 55). In the latter case, the mediator acts by an electron relay between the enzyme and the electrode. Enzyme electrodes that use redox mediators present unique advantages over conventional biosensors. The measurements are insensitive to oxygen fluctuation and may be performed under anaerobic situations. The linear concentration ranges are extended, and because of the low redox potential of the mediator, the indicator electrode may be polarized at low potentials, thus minimizing the risks of interferences from other electroactive species (56).

3.1.3. THIRD-GENERATION GLUCOSE ELECTRODES

The third generation of enzyme electrodes uses direct electrical communication between the redox centers of the enzymes and the electrode surface. This con-

TABLE 4

Chemical Structure of Some Redox Mediators



Note: A, tetrathiafulalene (TTF); B, tetracyanoquinodimethane; C, ferricinium; D, *N*-methylphenazinium (NMP^+); E, Meldola blue.

figuration may be regarded as a mediatorless enzyme electrode. Except for the organic metal electrode in the presence of cytochrome b2, peroxidase (48), only a few successful results have been reported due to the inherent difficulties related to the complex structure of proteins (57, 58). Moreover, this class of enzyme electrodes is difficult to identify in the literature since the exact nature of the electrode mechanism, that is, whether the enzyme is oxidized by direct electron transfer to the electrode or whether it is a mediated process or simply detects liberated hydrogen peroxide, is often not defined.

3.2. Potentiometric Urea Electrode

Potentiometric enzyme electrodes are analytical devices that incorporate a biological sensing element intimately associated with a suitable sensing device, for example, pH-glass, ion-selective (ISE), solid state-pH, and redox electrodes, or gas electrodes (Table 6). In the potentiometric approach, the electrode interface senses the steady-state concentration (activity) of some species (ions) produced or consumed by the enzymatic reaction. The potentiometric transducers ISE and ion-selective field effect transistors (ISFET) exhibit a logarithmic dependence on the concentration of the substrate. Although the nature of the process involved at the biological site of potentiometric biosensors may be quite distinct

(e.g., biocatalysis, immunoreaction, chemoreception), the measurable signal is a potential change due to the biological reaction. The first potentiometric biosensor was developed by Guilbault and Montalvo (59) for urea in 1969. Since then, many different potentiometric enzyme electrodes have appeared in the literature (1, 8, 26). Originally, ion-selective electrodes, described as membrane sensors, served as the physical transducer. The potential, which develops across an ion-selective membrane separating two solutions, is measured versus a reference electrode at virtually zero current. Urea is degraded in the urea electrode by urease:

$$H_2NCONH_2 + H_2O \xrightarrow{\text{Urease}} 2NH_3 + CO_2 \quad [8]$$

$$NH_3 + H_2O \rightleftharpoons NH_4^+ + OH^- \quad [9]$$

$$CO_2 + H_2O \rightleftharpoons HCO_3^- + H^+ \quad [10]$$

and many different sensors can be used to follow the overall reaction (see also Table 6).

The first electrode for urea was prepared by immobilizing urease in a polyacrylamide gel on nylon or Dacron nets. The nets were placed onto a Beckman electrode (NH_4^+ selective) (59). In a later development, the electrode was improved by covering the enzyme gel layer with a cellophane membrane to prevent leaching of urease into the solution (60). The urease electrode could be used for 21 days with no loss of activity.

In attempts to improve the selectivity of the urea determination, a silicon rubber-based nonactin ammonium ion-selective electrode was used together with immobilized urease in polyacrylic gel (61). An additional improvement in the selectivity was obtained by polymerizing urease directly onto the surface of an Orion ammonia gas membrane by means of glutaraldehyde (62). Sufficient ammonia was produced in the enzyme reaction layer, even at pH values as low as 7–8, to allow direct assay of urea in the presence of large amounts of Na^+ and K^+. A response time of 2–4 min was observed. A urea electrode using physically entrapped urease and a glass electrode to measure a pH change in the solution was also described (63). The response time of the electrode to urea was about 7–10 min, with a linear range of 50 μM to 10 mM.

Another urea electrode uses a carbon dioxide sensor covered with urease to measure the second product of the urea–urease reaction, HCO_3^-. Na^+ and K^+ had no influence on this electrode and the linear range was 0.1–10 mM (64).

Wire that senses pH changes such as antimony metal can be coated with urease (65) and used to determine urea in pure blood (66). Covering the antimony metal with a gas perm-selective membrane (67) improves the selectivity. The microsensors respond from 0.1 to 10 mM urea in 30–45 s. This ammonia sensor has a faster baseline recovery than commercial gas membrane electrodes.

Enzyme electrodes that use urease attached with glutaraldehyde and a cation-selective glass electrode sensor have a range of 10 μM to 0.1 M (68). They have

been successfully used for enzyme inhibition studies by various pollutants (69).

Recently original all solid-state electrodes for NH_4^+ were successfully combined with urease for the assay of urea (70, 71). These electrodes consist of a conductive resin (epoxy + graphite) covered by a nonactin-PVC matrix. They offer interesting commercial advantages over membrane electrodes with internal solution and reference electrode.

A new development in the field of potentiometric enzyme sensors came in the 1980s from the work of Caras and Janata (72). They describe a penicillin-responsive device which consists of a pH-sensitive, ion-selective field effect transistor (ISFET) and an enzyme-immobilized ISFET (ENFET). Determining urea with ISFETs covered with immobilized urease is also possible (73). Current research is focused on the construction and characterization of ENFETs (27, 73). Although ISFETs have several interesting features, the need to compensate for variations in the pH and buffering capacity of the sample is a serious hurdle for the rapid development of ENFETs. For detailed information on the principles and applications of ENFETs, the reader is referred to several recent reviews (27, 74) and Chapter 8.

3.3. Electrode Characteristics

Conventional electrodes generally employ macroscopic membranes to solve problems associated with enzyme immobilization, interference, and surface fouling by the biological environment. While these devices may be used successfully for some applications, they suffer from several limitations, such as slow and complex diffusion path for reactant and product and reproducible enzyme immobilization. Decreasing the thickness of the biocatalyst layer provides a significant improvement in these electrodes. The enzyme may be physically adsorbed onto "porous" platinum and carbon electrodes (75–78), or, better, chemically attached to solid electrodes possessing functional groups (79, 80). The incorporation of the enzyme into the electrode matrix may also be advantageous (81, 82). Such electrode configurations allow optimum molecular proximity between the enzyme active sites and the electrochemical surface and minimize mass transfer through the immobilized biocatalyst layer. Improvement in the control of the immobilization procedure is achieved by entrapping the enzyme into the network of electropolymerized matrices (83–86). This technique allows microfabrication and more precise control of the film thickness, homogeneity, and reproducibility. Further studies are oriented toward the use of appropriate perm-selective polymer film membranes (protein or bacteria repellent, biocompatible, etc.) (87, 88).

4. IMMOBILIZATION PROCEDURES

Physical localization of the enzyme with high activity in a thin layer over the electrode surface can be achieved by both covalent and noncovalent procedures. In

its simplest mode, the immobilization may be performed by simply retaining the "soluble" enzyme as a thin film over the electrode surface using a semipermeable membrane (39, 93–94). This procedure is readily performed, but the resulting probe often shows poor stability and requires large quantities of enzyme. Electrodes with physically or chemically immobilized enzymes exhibit greater stability and less interference. The following are the preferred enzyme immobilization procedures:

1. Entrapment in an inert polymer matrix over the electrode.
2. Cross-linking the enzyme to itself or to other macroscopic particles (protein) with bifunctional coupling reagents to localize the immobilized enzyme as a thin layer over the electrode.
3. Coupling the enzyme directly onto the electrode surface or to water-insoluble membranes maintained over the electrode, with coupling reagents.

Stable sensors can be prepared by the simple and straightforward physical adsorption of the enzyme onto a variety of electrode material (75–78, 121).

The choice of the appropriate mode of immobilization is significant, since molecules of all sizes can interact with the adsorbed and chemically immobilized enzyme, whereas large molecules are not able to diffuse into the biocatalytic layer of the entrapped enzyme. Thus, for the assay of large substrates, such as proteins, an attached enzyme must be used. Either form of the enzyme could be used for the assay of small substrates, such as glucose or urea. Tables 5 and 6 illustrate the diversity of the immobilization techniques for glucose and urea electrodes. Many laboratories apply their own recipe for enzyme immobilization (89); others use commercially available pre-activated membranes (90–92). No immobilization mode has emerged that is best for all applications. Since each enzyme is unique in terms of structure and activity, the general rule is to ensure that the treatment will not inactivate functional groups of the enzyme that are essential for its catalytic activity.

An important aspect of enzyme immobilization is the fact that the thinner and more active the enzyme layer, the faster and more sensitive will be the response. Generally, an outer protective perm-selective membrane must be cast over the sensor head, and the best immobilization will be a thin, homogeneous enzyme layer in close proximity with the transducer. The outer membrane prevents the sensor from fouling, excludes interfering substances from reaching the electrode surface, and controls the diffusion of analyte and co-substrate (oxygen). By proper selection of the covering membrane, an extended linear range may be observed with oxidase-based sensors. This is explained by the fact that the membrane acts as a diffusion barrier to reduce the local substrate concentration while maintaining sufficient oxygen for the enzyme reaction.

The advantages of diffusion-control enzyme electrodes over devices controlled by the enzymatic reaction (kinetic-control) are that the linearity is increased above the K_m and the response is no longer dominated by the enzyme reaction. This implies that the enzyme electrode is less sensitive to pH and tem-

TABLE 5

Various Glucose Electrodes

Modes of Immobilization	Modes of Detection	References
Physical entrapment between membranes	Amperometry	39, 93, 94
Physical entrapment into inert supports		
Cellulose acetate	Amperometry	95
Gelatin	Amperometry	96–98, 103
Polyacrylamide	Amperometry	49, 99–102
	Potentiometry	122, 123
Polyurethane	Amperometry	89, 103
Polyvinyl alcohol	Amperometry	104, 105
Polyvinyl chloride	Potentiometry	106, 107
Carbon composite	Amperometry	50, 81, 82, 108–114
Redox gel	Amperometry	38
Lipids	Amperometry	115, 116
Conducting polymers	Amperometry	36, 83–86, 118–120
	Potentiometry	117
Chemical cross-linking with an inert protein		126
GA + Collagen	Amperometry	68, 128–132
GA + BSA	Amperometry	127, 106
GA + Gelatin	Potentiometry	133, 134
	Amperometry	
Covalent linking to water insoluble carrier		
Nylon mesh	Amperometry	135–137
Collagen	Amperometry	141
Cellulose acetate	Amperometry	142, 187, 188
Pig small intestine	Amperometry	138
Attachment onto the electrode surface		
Physical adsorption	Amperometry	32, 75–78, 121, 84, 148–150
	Potentiometry	144–147
Covalent linking	Amperometry	152, 154–156, 58, 79, 80, 179

perature and that there is a reduced effect of bulk solution stirring and viscosity on the electrode signal. Care must be exercised, however, to ensure a good response time, since a thick membrane can cause prolonged response time.

4.1. Physical Entrapment

Entrapping an enzyme in a gel, such as gelatin, starch, or polyacrylamide, is a physical and mild method of fixing protein because the polymer is allowed to form cross-links in the presence of the enzyme (95–102). Several enzymes have been entrapped in polyacrylamide gels, for example, glucose oxidase, catalase, lactic dehydrogenase (LDH), amino acid oxidase, and glutamate dehydrogenase

TABLE 6

Various Urea Electrodes

Electrode	Immobilization	References
pNH_3	Cyanuric chloride cross-linking with polyvinyl alcohol-*g*-butyl acrylate	204
pNH_3	GA + Teflon or polypropylene	62, 139
pNH_3	Agar gel or GA + cellulose acetate	205
Glass NH_4^+	Polyacrylamide gel	60
pCO_2 or glass NH_4^+	GA + BSA	68
Silicon rubber-based nonactin NH_4^+	Polyacrylic gel	61
PVC-based nonactin NH_4^+	GA + BSA + amino-silanized polyester	206
PVC-based nonactin NH_4^+	GA	207
pH − glass	Polyacrylamide - hydrazide + glyoxal	102
Sb + gas membrane	GA + BSA	67
Graphite - epoxy PVC - nonactin NH_4^+	Acrylamide gel	70, 71

(GDH). The gels show little loss in activity after 3 months of storage at 0–4 °C (99).

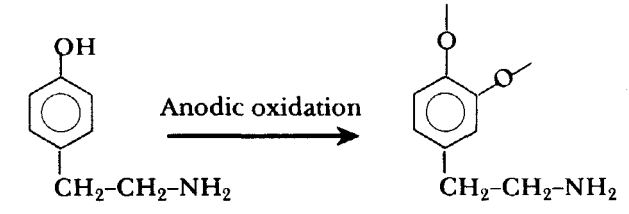
A comparative study of the immobilization of cholinesterase and urease in starch gel, polyacrylamide, and silicone rubber has been reported (100). Physically entrapping the enzyme in the polyacrylamide gel gives the best results. The silicone rubber polymerization inactivates the enzyme (80% loss of activity). In starch gel the enzyme is too weakly held, and much of the enzymatic activity is lost due to leeching. The stability of the enzymes in polyacrylamide is better, particularly in the case of urease (80 days). However, some enzyme is lost during acrylamide polymerization (10–25% loss). An additional problem is that riboflavin and $K_2S_2O_8$, catalysts for the polymerization, can interfere in the assay, depending on the mode of detection. The technique has recently been successfully applied to the determination of urea in diluted plasma using an urease-immobilized glass pH electrode (101).

Directly coating the surface of a glass pH electrode with a thin layer ($\simeq 50$ μM) of entrapped enzyme is possible by a controlled chemical cross-linking of a prepolymerized linear chain of polyacrylamide–hydrazide by glyoxal (102). Acetylcholine esterase, urease, and penicillinase have been successfully immobilized in this manner and the sensors exhibit surprisingly long shelf life (more than 6 months for acetylcholine esterase) as well as short response times and wide linear responses. Polyurethane is used to entrap a great variety of enzymes (89, 103). Polyvinyl alcohol gels are also used to entrap urease; the resulting membranes are mounted over a platinum anode (104). Recently, stable films of the same polymer containing GOD were obtained by exposing the electrode surface to gamma radiations (105). This technique offers important advantages over chemical and UV cross-linking polymerization techniques, provided that the radiation

causes no damage of the biological component. Glucose oxidase is immobilized in plasticized polyvinyl chloride, either alone or with peroxidase, and the layers are coated onto an iodide ion-selective electrode (106, 107). Comparing the performance of the GOD immobilized PVC membranes to GOD chemically immobilized with glutaraldehyde-bovine serum albumin membranes under identical conditions shows that the former exhibits higher sensitivity, but suffers from shorter lifetimes: 7 days compared to more than 2 weeks for the chemically immobilized GOD.

To promote access of the substrate to the enzyme-active site, the enzyme is entrapped within the electrode matrix itself (81, 82, 100–110). In the first attempt, thin particles of graphite were incorporated into the enzyme–polyacrylamide gel loaded with an electron transfer promoter, ferrocene (82). A number of authors have advantageously used this technique to immobilize enzymes, either alone (81) or with a redox mediator (108–110). The enzymes GOD (100–111), tyrosinase (112), sulfite oxidase (113), alchohol dehydrogenase (114), and glycolate oxidase (50) have been immobilized in carbon paste (81, 108, 110, 112, 113) or composite graphite–epoxy matrices (109, 111). The resulting probes provide a reservoir of enzyme in a stable "dry" state, allowing easy renewal of the electrode surface. Recently, an elegant entrapment of GOD within a gel structure, containing redox-active sites to promote electron relays between the enzyme and the electrode, was reported (55). Localizing the "soluble" enzyme among lipid layers with the hope of improving the enzyme stability and activity has been attempted (115, 116). An additional advantage is perm-selective diffusion of substrates through the lipidic coating (115).

The enzyme may also be attached to or entrapped in pre-formed conductive polymers on the surface of microelectrodes. Pyrrole and its derivatives (83–86, 117, 120), tyramine (118)



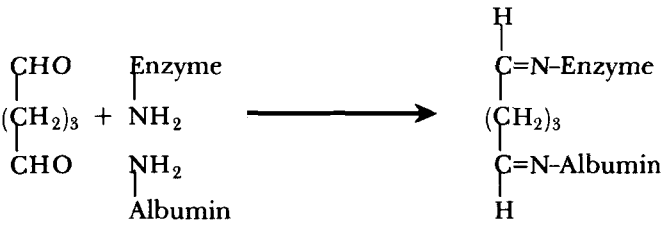
Tyramine Polytyramine [11]

1,2-diamminobenzene (119), aniline (149), and indole (150), are successfully polymerized onto various electrodes for enzyme entrapment. These polymers may additionally contain attached redox mediators to accelerate the electron transfer kinetics (53).

4.2. Chemical Immobilization

4.2.1. CROSS-LINKING WITH A BIFUNCTIONAL REAGENT

Enzymes may be immobilized with bifunctional reagents by several different methods: direct internal cross-linking of enzymes or cross-linking of enzymes with inert proteins such as albumin, gelatin, or collagen (126–134). The most commonly used combination of reagents is glutaraldehyde (GA) as the bifunctional reagent and bovine serum albumin (BSA) as the inert material:



[12]

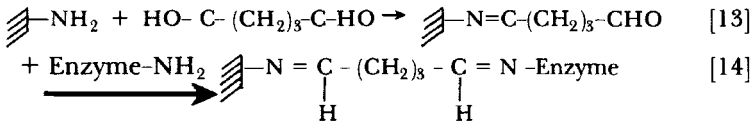
The technique is simple and fast and allows control of the physical properties and particle size of the final product. Nevertheless, care must be taken in the selection and storage of glutaraldehyde to ensure reproducible enzyme immobilizations (123). Buffers containing reactive amino groups must be avoided. Several other bifunctional reagents are more or less successful. The most common are hexamine diisocyanate, trichloro-*s*-triazine, and diphenyl-4,4′-dithiocyanate-2,2′disulfonic acid (8, 124, 125).

4.2.2. COVALENT BINDING TO WATER-INSOLUBLE MATRICES

Binding the enzyme covalently to water-insoluble matrices provides high stability, improved catalytic efficiency, and the possibility of using a wide range of carriers, including electroconductive ones. Carrier composition is of primary importance since it determines the type of enzyme attachment and the durability of the resulting probe. Corrosion or dissolution of the support can shorten the enzyme's operational half-life, either by enzyme loss or by deactivation of the enzyme caused by inhibition of the catalytic site with soluble corrosion products. Moreover, the carriers are chosen on the basis of their solubilities, functional groups, surface area, swelling, and hydrophobic or hydrophilic nature.

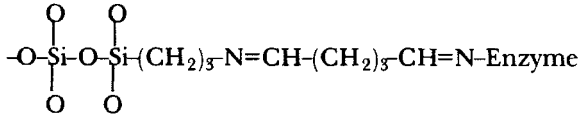
Essentially, three types of carriers are used: inorganics and natural and synthetic polymers. The binding reactions must proceed under conditions that do not cause enzyme denaturation. Enzymes are linked through functional groups that are not essential for catalytic activity. The amino acid residues that are the most suitable for covalent binding are the α- and ε-amino groups, the β- and γ-carboxyl groups, and the phenol ring of tyrosine. A large number of methods for covalent coupling enzymes to water-insoluble carriers are available. Attaching enzymes to the activated matrix via glutaraldehyde is simple and inexpensive:

Activation



Enzymes have been attached to a nylon matrix (135–137), a pig intestine (138), the hydrophobic membrane of a gas-selective sensor (139), and controlled pore glass (140). Recent comparative studies of the coupling agents GA and benzoquinone support the preferential use of benzoquinone for binding GOD to nylon mesh (137), or to cellulose acetate membranes (141) with lysine (137). Both investigations report robust electrode behaviors with respect to prolonged exposure to glucose, while lifetimes of the membrane electrodes were ca. 3 months.

Generally, the carriers must be activated to be suitable for covalent attachment of the enzyme molecule, by introducing or liberating a functional group that undergoes a coupling reaction with the enzyme under mild conditions. Arginase and urease are simultaneously immobilized onto controlled pore glass beads and deposited on the surface of an ammonia-sensing probe for the determination of arginine (140). In the electrode assembly, the enzyme beads are trapped between a nylon membrane and the ammonia-sensing membrane. The resulting probe stored at 4 °C is stable for at least 41 days. The immobilization procedure involves activation of the glass beads with an alkylamine derivative and subsequent coupling of the enzymes with GA giving the following enzyme bead formula:



Chemically binding enzymes to nylon net is very simple and gives strong mechanically resistant membranes (135). The nylon net is first activated by methylation and then quickly treated with lysine. Finally, the enzyme is chemically bound with GA. The immobilized disks are fixed directly to the sensor surface or stored in a phosphate buffer. GOD, ascorbate oxidase, cholesterol oxidase, galactose oxidase, urease, alcohol oxidase (135), and lactate oxidase (142) have been immobilized by this procedure and the respective enzyme electrode performance has been established.

Collagen membranes also bind a variety of enzymes (141). The binding procedure is particularly mild because the enzyme never comes in contact with the chemical reagents, avoiding all risks of denaturation. Such membranes, however are too thick and too fragile, especially at 37 °C, to be recommended for in vivo applications of enzyme electrodes (142). Several commercial preactivated membranes are available that provide simple and fast procedures for immobilizing membranes (90–92, 143). The stability of the enzymatic membranes were excellent: More than 400 assays were performed within 50 days.

4.2.3. DIRECT ATTACHMENT ONTO THE ELECTRODE SURFACE

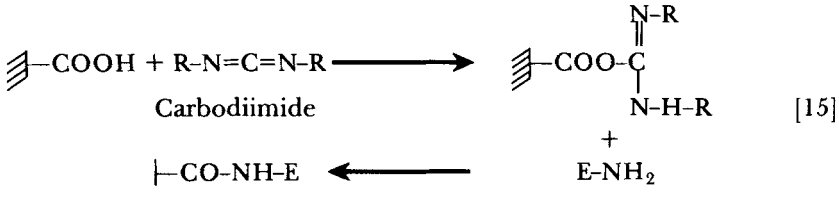
Attaching the enzyme directly on the electrode surface is expected to improve electrocatalytic efficiency and response and improve the reproducibility of immobilization (147). Metallic (122, 144, 145) and carbonaceous (146) enzyme electrodes develop potentiometric responses to H_2O_2 produced by the enzymatic reaction. Unfortunately, the signal is markedly dependent on the redox surface of the electrode and thus on the electrode pretreatments (which are quite difficult to reproduce).

4.2.3.1. Physical Attachment. Carbonaceous materials are effective in adsorbing enzymes because of the presence of various functional groups on their surface and their porosity. However, the adsorption is reversible and the probes often exhibit poor stability, losing their activity within a few days (147). Nevertheless, viable sensors are reported (75–78, 121). Improved adsorption of enzymes is achieved by increasing the surface area and porosity of the electrode by (1) depositing platinum (75, 78, 148) or palladium/gold particles (121) on solid electrodes, (2) modifying the electrode surface with conducting polymers (83, 84, 149, 150), or (3) electrochemical attachment of proteins (BSA) onto the electrode surface (151).

4.2.3.2. Chemical Attachment. The initial step in chemically attaching enzymes to electrodes involves an activation (derivatization) of the support to introduce appropriate functional groups, such as carboxy, phenol, and quinone-like structures. This step is critical and must be controlled rigorously since the greater the number of functional groups, the higher will be the amount of immobilized enzymes attched and the better the final electrode activity. The electrode surface may be activated by different ways:

1. Oxidation by heat, by radiofrequency plasma, chemically, or electrochemically (cathodic and extreme anodic polarization cycles). The resulting surface can be linked to enzymes directly or with a coupling agent (58, 79, 80, 152, 153).
2. Silanization of the activated surface and introduction of an amine terminated spacer arm (154, 179).

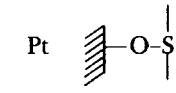
The coupling is generally accomplished by GA or carbodiimide, which leads to intra- and interenzyme cross-linking in addition to the desired enzyme support cross-linking:



[15]

The resulting mono or plurimolecular layers maintain approximately 75% of the initial activity after a month's use.

Carbonaceous substrates (graphite and glassy carbon) are generally preferred because of their mechanical, chemical, and electrochemical properties. Excellent results are also obtained by chemically modified platinum (154, 156, 179) and tin(IV) oxide electrodes (155). For example, glucose oxidase has been successfully immobilized by cross-linking the enzyme with BSA and GA onto an electrochemically oxidized platinum surface, with silanization using 3-aminopropyltriethoxysilane:



[16]

Detecting H_2O_2 by poising the electrode at a potential of +0.7 V against the saturated calomel electrode (SCE) gives linear graphs from 0.1 μM to 2 mM. The electrode response is fast, and the device is stable for more than one month. Analytical results for glucose in human control serum samples were in agreement with the manufacturer's data (156).

5. ENZYME ELECTRODE CHARACTERISTICS

5.1. Stability

The lifetime of an enzyme electrode is characterized by both its storage and its operational stability. Because of their hybrid structure, factors affecting electrode stability and enzyme stability are considered. Potentiometric ion-selective electrodes possess great stability, but in case of gas-sensing devices, the internal filling solution must be replaced regularly. Despite their unique conception, gas-sensing electrodes respond to other small molecules that diffuse across the membrane barrier. Indeed, interfering volatile gases, such as organic amines and CO_2, in the analyzed sample produce erroneous and abnormally high responses. Experimental verification of the responses of gas sensors to interfering gases and several theoretical predictions are reported (157–160). The selectivity of a CO_2 probe is time and concentration dependent (157). In the cases of NH_3 sensors, the selectivity is determined by the acidity and basicity of the interferents rather than by the volatility (158, 159). For homogeneous plastic films, the solubility of the gas in the membrane material must be considered (160).

The stability of amperometric sensors is affected by progressive surface fouling by the reaction products, especially when operating at high potentials. Redox mediators can be used to minimize the rate and extent of surface fouling. Monitoring the reaction by pulsed amperometric detection techniques (161) or kinetic methods (162) is also possible. Instead of waiting until an equilibrium current is reached, the rate of change of current ($\Delta i/\Delta t$) can be measured and equated to the concentration of the substrate. Thus, the time during which the reaction occurs is short, minimizing the amount of end product formed (161, 162).

The mode of immobilization, as well as the source and extent of purification of the enzyme, are important factors in determining the lifetime of the biocatalyst. Generally, the lifetime of a "soluble" enzyme electrode is about one week or 25–50 assays, and the physically entrapped polyacrylamide electrodes are satisfactory for about 50–100 assays, depending primarily on the degree of care exercised in the preparation of the polymer. The chemically attached enzyme can be kept for years, if used infrequently. In frequent use, the GOD electrode has a lifetime of over one year and can be used for over 1000 assays. For l-amino acid oxidase or uricase (100) biosensors, about 200–1000 assays per electrode can be obtained, depending on the immobilization technique.

With amperometric sensors, high operating potentials and products of the reaction (H_2O_2) may inactivate the enzyme. Thus, it is advantageous to measure the electrode response kinetically and to minimize the time that the biosensor is in contact with the substrate (162). Although the electrode can be stored at room temperature, all enzyme-based electrodes should be kept in the refrigerator (around 5 °C) and covered with a dialysis membrane to prevent the contamination of bacteria, which tend to feed on the enzyme, destroying its activity. Another factor affecting the stability of some enzyme electrodes is the leaching of loosely bound cofactor from the active site and the redox mediator if any is present (163). Elegant solutions to these problems were provided by constructing pen-sized disposable biosensors, which are already commercially available (45, 164).

5.2. Response Time

A bioelectrode functioning optimally has a short response time, which is often controlled by the thickness of the immmobilized enzyme layer rather than by the sensor as well as many other factors (see Table 7). The biosensor response time depends on (1) how rapidly the substrate diffuses through the solution to the membrane surface, (2) how rapidly the substrate diffuses through the membrane and reacts with the biocatalyst at the active site, and (3) how rapidly the products formed diffuse to the electrode surface where they are measured. Mathematical models describing this effect are thoroughly presented in the biosensor literature (5, 68).

The amount of enzyme, either in a pure or crude form, also affects the speed of response of the electrode. Yet, compromises must be made between the increase in enzyme activity and the concomitant increase in membrane thickness, which affects the rate of electrode response. As the amount of enzyme is increased, a shorter response time is observed, until an optimum level is reached. Further increase in the amount of enzyme tends to diminish the response time due to a thickening of the membrane layer and an increase in the time required for the substrate to diffuse through the membrane. Generally, using enzyme with the highest specific activity gives the thinnest membranes and the most rapid kinetics.

TABLE 7

Factors Affecting the Response Time of an Enzyme

Stirring rate of the solution
Concentration of the substrate, 0.1 M > 1 mM > 10 μM
Concentration of the enzyme
pH optimum
Temperature (most effect on rate)
Dialysis membrane

Note: A fast response is defined as a low response time.

5.3. Selectivity

5.3.1. INTERFERENCE IN THE ELECTRODE PROBE

Ideally, the sensor used to sense the biocatalyzed reaction should not react with other substances in the sample. This requirement is not always met using either potentiometric or amperometric methods. For example, immobilized urease electrodes operating with a cation glass sensor measuring the NH_4^+ are inadequate for blood and urine assays because they also respond to Na^+ and K^+ (59, 60). However, a glass electrode sensor (165) or, better, a solid antibiotic nonactin electrode (61) gives more selective response. The latter has a selectivity of NH_4^+/K^+ of 6.5 and NH_4^+/Na^+ of 0.075.

Amperometric devices using solid electrodes operate at a judiciously selected potential, but are still subject to interferences by electroactive substances. The Clark-type oxygen electrode, due to its unique design (surface covered with a gas-permeable membrane) is very selective. The detection of H_2O_2 at positive potentials (+0.6 V vs. Ag/Ag^+) is perturbed in many analytical applications by easily oxidizable molecules such as ascorbic acid, uric acid, acetaminophen, and iron(II) (40, 42–45). Lowering the operating potential is achieved by using redox mediators (40) or appropriate inorganic catalysts, such as Ni–cyclam complexes (166).

5.3.2. INTERFERENCE WITH THE BIOCATALYST

Such interference falls into two classes: competitive substrates and substances that either activate or inhibit the enzyme. With some enzymes, such as urease, the only substrate that reacts at reasonable rate is urease; hence, the urease-coated electrode is specific for use (59, 165). Likewise, uricase acts almost specifically on uric acid (167), and aspartase on aspartic acid (8, 168). Others, such as penicillinase and amino oxidase, are less specific (63, 169, 170). Alcohol oxidase responds to methanol, ethanol, and allyl alcohol (171, 172). Hence, in using electrodes of these enzymes, the analyte must be separated if two or more are present (172). Assaying L-amino acids by using either the decarboxylative or the deaminating enzymes, each of which acts specifically on a different amino

acid, is an attractive alternative (64). Enzyme electrodes of this type are known for L-tyrosine, L-phenylalanine, L-tryptophan, and others.

The activity of the enzyme is also strongly affected by the presence of inhibitors. Fluoride ions inhibit urease (173) and oxalate ions inhibit lactate oxidase (174), but the major inhibitors are heavy-metal ions, such as Ag^+, Hg^{2+}, Cu^{2+}, organophosphates, and sulfhydryl reagents (*p*-chloromercuribenzoate and phenylmercury(II) acetate), which block the free thiol groups of many enzyme active centers, especially oxidase (69). Inhibiting the enzyme electrodes makes it possible to quantify the inhibitors themselves (69), for example, H_2S and HCN detection using a cytochrome oxidase immobilized electrode (176).

5.3.3. INFLUENCE OF pH

The influence of pH on the response of the immobilized enzyme electrode must be considered by its effect on both the immobilized system and the detector. Every enzyme has a maximum pH at which it is most active and a certain range of pH in which it demonstrates reactivity. Moreover, when the enzyme is immobilized, the optimal pH may shift, depending on the nature of the carrier (8). According to the Nernst equation, the potential at the electrode of a given reaction is governed by the pH of the solution layer. A number of redox processes are pH dependent, for example, H_2O_2 oxidation; therefore, rigorous control of pH is required for optimal response. However, the apparent pH in the vicinity of the immobilized biocatalytic layer may differ from the solution pH (175) and usually a high buffer capacity is applied to minimize this effect. For the fastest and most stable and sensitive response, the pH optimum of the enzyme system should be chosen as the working pH. This is not always possible, because the electrode may not respond optimally at the pH of the enzyme reaction. Thus, a compromise is often necessary between these two factors. However, the pH of the biocatalyst system should not be forced to conform with the pH requirements of the sensor.

5.3.4. QUANTITATIVE DETERMINATION AND LIMITS OF DETECTION

All enzyme electrodes measure substrate in the concentration range of 0.1 mM to 0.1 M, with detection limits as low as 1 μM. In potentiometry, the slope of the calibration curve of potential versus log [concentration] is Nernstian in the linear range, that is, with a slope of 59.1 mV/decade. All curves level off at high substrate concentrations, as predicted by the Michaelis-Menten equation, uhich states that the reaction becomes independent of substrate at high substrate concentration. A leveling off of the curve at low substrate concentration is also observed, due to the limit of detection of the electrode sensor used (Fig. 6). Higher sensitivities may be achieved by using amperometric devices, by applying the substrate amplification mode, or by performing immunoelectrochemical assays (32, 89).

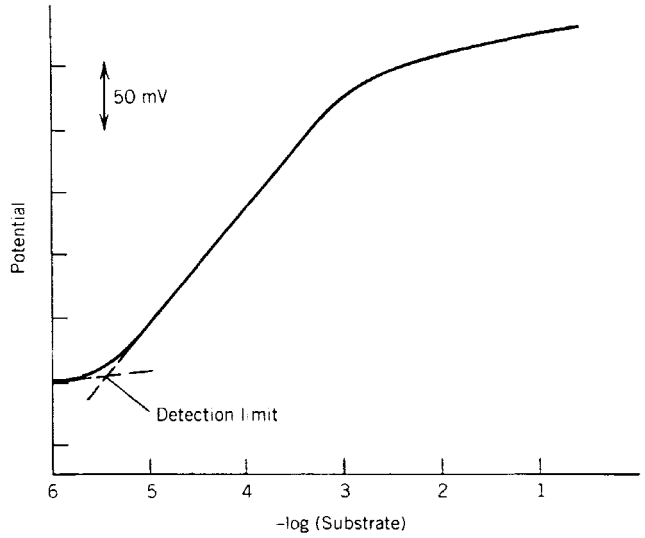


Fig. 6. Typical response curve of a potentiometric enzyme electrode.

6. APPLICATIONS OF ENZYME ELECTRODES

6.1. Glucose and Carbohydrates

The construction of glucose electrodes is rapid and simple, requiring the entrapment of the enzyme between an inner perm-selective membrane and an outer protective and semipermeable membrane (e.g., cellulose acetate, polyurethane, nylon) at the tip of the probe. When the biosensor is immersed in a solution of glucose, the glucose diffuses through the outer membrane into the enzyme layer and reactions proceed. The inner perm-selective membrane, which must be extremely thin (1–2 μM) to allow rapid responses, is selected depending on the nature of the species to be detected, that is, a hydrophobic (polypropylene, Teflon), oxygen-permeable or a cellulose acetate H_2O_2 perm-selective membrane. Several improvements in the perm-selective and protective properties of membranes are obtained with new materials, such as perfluorosulfonic acid polymers (76, 87), or by directly linking conductive polymers (53, 86, 116).

Although oxygen or H_2O_2 monitoring enzyme electrodes are successfully applied under certain circumstances, some problems are encountered when they are applied to glucose analysis in whole blood, in vivo or in fermentation broths (where oxygen concentration is usually low and is subject to large variations) (111, 177, 178). Various methods are used to increase the oxygen concentration within the biocatalyst layer:

- Using highly oxygen soluble polymers, such as gelatin (96).
- Using a very thin enzyme layer covered by a rubber membrane of high oxygen permeability (179).

- Co-immobilizing catalase and GOD. The former destroys H_2O_2 with liberation of oxygen, which reduces the total demand of oxygen by 50% and protects the enzyme GOD from deactivation by H_2O_2 (182).
- Generating oxygen electrochemically at the sensor tip (130).
- Limiting glucose diffusion without affecting oxygen diffusion by careful selection of membranes (88, 129, 180).

Less popular, glucose dehydrogenase-based electrodes monitor glucose potentiometrically (181) or amperometrically (183–185). In most instances, glucose dehydrogenase (GDH) systems are also dependent on the oxygen tension of the sample, and redox mediators (Meldola blue) are incorporated (186). Comparative performances of the GOD- and GDH-based electrodes are available for monitoring glucose in baker's compressed yeast production (185).

A serious limitation when working in complex media such as blood is the rapid loss of response due to a coating of the electrode membrane by blood constituents (platelets, fibers, lipids, etc.). Minimizing these undesirable effects is possible by (1) using repellent membranes, either negatively charged (129, 180, 187) or hydrophobically treated (88, 129, 133, 188), (2) using anti-platelet agents in immobilizing the enzyme in cellulose acetate membranes containing heparin (189), or (3) by reducing the flow stream toward the electrode (i.e., sample dilution in FIA manifolds) (133).

Galactose is determined by immobilizing the enzyme galactose oxidase at an oxygen electrode (190) or at a micro-platinum electrode operating anodically to monitor H_2O_2 formation (191, 192). The enzyme is immobilized on collagen membranes (193) or cellulose acetate membranes, and the probe is applied selectively to plasma and whole blood determinations (191, 194).

Determining maltose is possible by using chemically activated collagen membranes, and by testing three different modes of enzyme co-immobilization: asymmetric coupling, random co-immobilization, and two membranes placed one on the other (193). The two enzymes were glucoamylase and GOD. Glucoamylase was immobilized on the membrane surface exposed to the bulk phase into which the maltose-containing samples were injected. The hydrolysis of maltose liberates glucose, which is oxidized at the inner face with immobilized GOD. Extended calibration curves were observed from 0.2 μM to 4 mM. The enzymes glucoamylase and GOD were also embedded in polyacrylamide gels and maintained over an oxygen electrode to monitor maltose. The resulting device was covered by a polyurethane foil to avoid starch interference and to increase the linear concentration range up to 250 mg/dL (195). A recent application for maltose used oligosaccharide dehydrogenase (ODH). This enzyme exhibits a broad substrate specificity: It catalyzes the oxidation of xylose, glucose, galactose, fructose, mannose, lactose, maltose, and so on, using organic dyes as the electron acceptor. Immobilizing ODH onto carbon paste containing *p*-benzoquinone makes it possible to sense maltose and lactose in milk and to assay α-amylase in serum standards. Due to its nonspecificity, the electrode is best applied after chromatographic separation of the carbohydrates (196, 197).

Maltose and starch were also determined by passing the sample through a packed bed containing immobilized amyloglucosidase and detecting liberated glucose with a glucose electrode (198). Using the three-enzymes, invertase, mutarotase, and GOD, in a three-enzyme electrode allows one to determine sucrose (21, 153, 193, 199):

$$\text{Sucrose} + H_2O \xrightarrow{\text{Invertase}} \alpha\text{-D-Glucose} + \text{D-Fructose} \quad [17]$$

$$\alpha\text{-D-Glucose} \xrightarrow{\text{Mutarotase}} \beta\text{-D-Glucose} \quad [18]$$

$$\beta\text{-D-Glucose} + O_2 \xrightarrow{\text{GOD}} \text{D-Gluconic acid} + H_2O_2 \quad [19]$$

Lactose electrodes have been constructed by asymmetric coupling of β-galactosidase and GOD (193). Lactose, sucrose, galactose, and glucose probes have been constructed by immobilizing the corresponding oxidase to nylon net and monitoring oxygen. The resulting single- or multienzyme device exhibits easy handling and high activities (135). Flow injection analysis (FIA) systems can be utilized to determine galactose in urine (200) and lactose in milk (201, 202) and foodstuffs (203).

6.2. UREA

As illustrated in Table 6 (see also Section 3.2), various enzyme probes are available for urea. Most of them monitor the enzymatically liberated ammonium ions or ammonia at basic pH values (204–207). Determining urea in urine or plasma samples is possible if care is taken to avoid interference of endogeneous ammonium ions. Urease is immobilized by two different techniques and used to determine urea in urine using an ammonia gas-sensing electrode (205). The enzyme is physically entrapped in agar gel or chemically immobilized on a cellulose acetate membrane with glutaraldehyde. Chemical immobilization requires more enzyme for optimal response due to some loss of enzyme activity, but the stability is superior to the physically entrapped enzyme. Since free ammonia interferes in urine samples, it is determined separately. As many as 1000 assays have been performed on one electrode with a coefficient of variation of 2.5% over the range 0.5 M to 10 mM. The throughput for serum was 20 assays/h, with excellent correlation with results obtained with the spectrophotometric diacetyl procedure (205). Urea can be determined in plasma (101), urine and serum samples (71, 102) by covering the tip of glass pH electrodes with a thin layer of immobilized urease physically entrapped in polymer gels or with antimony pH-sensitive electrodes covered with a chemically immobilized urease membrane (66).

6.3. Alcohols and Acids

6.3.1. ALCOHOLS

Enzyme electrodes for ethanol may be constructed by using either alcohol oxidases or alchohol dehydrogenases (ADH). With the former, either an oxygen electrode (208, 215) or a H_2O_2-detecting electrode can be applied (171). Ethanol is determined in a 1-mL sample over the range of 0–10 mg/100 mL, by poising the electrode anodically (171). Methanol seriously interferes unless the potential is set at negative values where the consumption of oxygen is monitored. Under these conditions 0.4 to 50 mg% of ethanol can be assayed with little interference (208).

Electrochemical sensors for ethanol are designed by constraining a dehydrogenase enzyme and NAD^+ onto the surface of a platinum electrode (209). Electrochemically activated carbon surfaces possess catalytic activity toward NADH oxidation at potentials as low as +0.2 V versus Ag/AgCl (at pH 7) (210).The electrodes are successfully applied for lactate, pyruvate and glucose-6-phosphate by using their respective dehydrogenase enzymes (211). Dehydrogenase-based enzyme electrodes are also available for monitoring ethanol in alchohol beverages. Here the electrode uses an appropriate redox mediator, hexacyanoferrate(III) (212). Others prefer to use the organic conductic salt $NMP^+/TCNQ^-$ as electrode material (see Section 3.1.2.) to oxidize NADH in the presence of ADH (213, 214).

TABLE 8

Various Lactate Electrodes

Enzyme	Electrode Measurable Species	Linearity	Application	References
LOD	H_2O_2	Up to 15 mM	Spinal fluid	223
LOD	H_2O_2	0 - 0.25 mM	Cell culture	221
LOD	H_2O_2	0.1 - 10 mM	Dairy products	231
LOD	O_2	0.25 μM - 0.25 mM	Milk, blood	230
LMOD	O_2	10 μM - 0.6 mM	Plasma	228
LMOD	O_2	0 - 0.8 mM	Whole blood	174
LMOD	O_2	0.3 - 25 mM	Physiological solution	232
Crude extract	O_2	0.1 - 10 mM	Dairy products, blood	233
LDH	NADH	Up to 15 mM	—	211
LDH	Hexacyanoferrate	0.05 - 7 mM	Milk, wine, blood	237
LDH	Ferrocytochrome c	Up to 6 mM	—	219

6.3.2. LACTIC ACID

The commercial availability of various lactate-specific enzymes and the high demand for lactate analysis for clinical and food processing led to the construction of numerous lactate enzyme electrodes (Table 8). Depending on the selected enzyme—lactate oxidase (LOD), lactate mono-oxidase (LMOD), or lactate dehydrogenase (LDH)—several mechanistic approaches may be applied:

$$\text{Lactate} + O_2 \xrightarrow{\text{LOD}} \text{Acetate} + CO_2 + H_2O \quad [20]$$

$$\text{Lactate} + O_2 \overset{\text{LOD}}{\rightleftharpoons} \text{Pyruvate} + H_2O_2 \quad [21]$$

$$\text{Lactate} + NAD^+ \overset{\text{LDH}}{\rightleftharpoons} \text{Pyruvate} + NADH + H^+ \quad [22]$$

$$\text{Lactate} + \text{2-Hexacyanoferrate(III)} \xrightarrow{\text{LDH}} \text{Pyruvate} + \text{2-Hexacyanoferrate(II)} \quad [23]$$

Measuring carbon dioxide is generally avoided because of the high bicarbonate content of blood and some dairy samples. Biosensors monitoring oxygen depletion are successfully applied in complex samples due to the inherent high selectivity of the Clark-type electrodes. For better sensitivities, monitoring H_2O_2 is preferred. Lactate dehydrogenase (LDH) can also be used, but to achieve good sensitivity, the equilibrium in equation 22 must be shifted in favor of the pyruvate side (229). Cytochrome-type LDH is used to determine lactate by following the reduction of hexacyanoferrate(III) (218) or ferriferrocytochrome c, and detecting the hexacyanoferrate(II) amperometrically (219). The potentiometric mode of detection is also based on equation 23, and the change in the ratio of the redox mediator couple is monitored (220).

The enzymes can be immobilized by a variety of methods: by physically entrapping them in gelatin (174, 220), polyacrylamide (174), or polyvinyl alcohol (222), by sandwiching them between membranes (219), or by chemically immobilizing them to GA-BSA (221) attached to nylon net (143, 230), cellulose acetate membranes (222, 223), or polyamide membranes (91), or directly onto the electrode surface (210, 224). Several strategies are employed to improve biosensor sensitivity: (1) adding a redox mediator such as phenazine methosulfate (225, 226), (2) using enzymes with high specific activities (143, 223), and (3) attaching the enzyme (LDH) onto the electrode surface (210, 224). The sensitivities generally achieved allow lactate determination in the range of concentrations between 5 μM and 5 mM. Even better sensitivities are obtained by combining the enzyme amplification principle given by

$$\text{Lactate} + D_{ox} \overset{\text{LOD or LDH}}{\rightleftharpoons} \text{Pyruvate} + D_{red} \quad [24]$$

$$\text{Pyruvate} + \text{NADH} + \text{H}^+ \overset{\text{LDH}}{\rightleftharpoons} \text{Lactate} + \text{NAD}^+ \qquad [25]$$

Amplification factors of 10 (227), 60 (217), and even 250 (222) are reported for microsamples of biological fluids.

6.3.3. PYRUVIC ACID

LDH enzyme electrodes follow electrochemically NADH consumption or NAD^+ formation (211). Electrochemically activated microcarbon electrodes can be utilized in conjunction with immobilized LDH to determine pyruvate in small volumes (50 μL) of cerebrospinal fluid within the concentration range 10 μM to 2 mM (234). The construction of pyruvate oxidase electrodes operating either at positive potentials (H_2O_2 detection) or at negative potentials (oxygen depletion) (92) is described by

$$\text{Pyruvate} + \text{H}_3\text{PO}_4 + \text{O}_2 \longrightarrow \text{Acetylphosphate} + \text{H}_2\text{O}_2 + \text{CO}_2 \qquad [26]$$

The immobilized enzyme requires thiamine pyrophosphate, calcium ions, and FAD for efficient activity (92, 235, 236). Concentrations as low as 1 μM have been determined (92).

6.3.4. URIC ACID

The enzyme electrodes for determining uric acid monitor reactants or products of the uricase catalyzed reaction:

$$\text{Uric acid} + \text{O}_2 + 2\text{H}_2\text{O} \overset{\text{Uricase}}{\longrightarrow} \text{Allantoin} + \text{H}_2\text{O}_2 + \text{CO}_2 \qquad [27]$$

Using platinum electrodes (167, 238) requires +0.6 V versus SCE to oxidize H_2O_2. However, this potential precludes selective measurements of uric acid because it is also oxidized at the electrode surface (167). Thus, to improve the selectivity, bienzyme amperometric devices using a redox mediator (hexacyanoferrate) have been constructed (239). The enzymes uricase and peroxidase are immobilized together and the hexacyanoferrate(III) is measured at 0.0 V versus Ag/AgCl. Alternatively, a carbon dioxide selective electrode is used for the detection of the enzymatically liberated CO_2 (240, 241).

6.3.5. SALICYLIC ACID

Salicylate is determined in blood serum using immobilized salicylate hydroxylase electrodes (243–245). This enzyme is a mixed function monooxygenase that converts salicylate to catechol in the presence of NAD(P)H and molecular oxygen:

$$\text{Salicylate} + \text{NAD(P)H} + \text{H}^+ + \text{O}_2 \rightarrow \text{Catechol} + \text{NAD(P)}^+ + \text{CO}_2 + \text{H}_2\text{O} \qquad [28]$$

Either the CO_2 formation is followed potentiometrically (243) or the O_2 consumption is measured amperometrically at an oxygen electrode (245). In the first method, the enzyme is physically immobilized with a dialysis membrane. The response is linear in the range 5–300 μg/mL of salicylate. The second technique uses chemically immobilized enzyme (GA + BSA) attached to a pig intestine mounted on the tip of the O_2 electrode. Samples containing from 10 μM to 2 mM salicylate were analyzed. An elegant microelectrode (244) has the enzyme and the cofactor immobilized in the electrode matrix (carbon paste) and the catechol formation is monitored at +300 mV versus Ag/AgCl. The electrode consists of a disposable strip, allowing measurements to be made on a drop of blood within 1 min.

6.3.6. OXALIC ACID

Oxalate is determined by using either oxalate decarboxylase (246–248) or oxalate oxidase (242, 249) immobilized electrodes. In the former, the CO_2 liberated and detected is proportional to the logarithm of the oxalate concentration. Linearity is reported from 0.2 to 10 mM, and the electrodes are stable for more than one month. Human control samples, spiked with oxalate, have been analyzed (246). Oxalate oxidase can be immobilized onto an O_2 electrode (242), an amperometric H_2O_2 sensor (246, 249, 250), and a potentiometric CO_2 probe (246):

$$\text{Oxalic acid} + O_2 \xrightarrow{\text{Oxalate oxidase}} 2CO_2 + H_2O_2 \qquad [29]$$

Oxalic acid in urine (242, 246) and in foodstuffs can be determined in the concentration range 5 mM to 0.1 M (250).

6.3.7. ASCORBIC ACID

Ascorbate electrodes for ascorbic acid determination in food (251), fruit juices (252) or pharmaceutical preparations (253) are based on the following reaction:

$$\text{Ascorbic acid} + \tfrac{1}{2}O_2 \xrightarrow{\text{AOD}} \text{Dehydroascorbic acid} + H_2O_2 \qquad [30]$$

The enzyme ascorbate oxidase (AOD) is immobilized over Clark-type oxygen electrodes. The biocatalyst is attached to polyamide nets with GA (252), cross-linked with collagen-glutaraldehyde (251) or albumin-GA (253), or linked to cellulose acetate membranes (253).

6.4. Creatine and Creatinine

Creatinine, the degradation product of creatine, is determined in serum with the picrate electrode, which uses the Jaffe method to potentiometrically monitor creatinine picrate. A prior separation step is required to remove interfering sub-

stances (254). An improved creatinine sensor is obtained by immobilizing creatinine deiminase onto an ammonia gas sensor. Direct reading of creatinine in the concentration range from 1 to 100 mg% is possible (254). A multienzyme electrode containing co-immobilized creatinine amidohydrolase, creatinine amidinohydrolase, and sarcosine oxidase on a cellulose acetate responds linearly up to 100 mg/L of creatinine and creatine in human serum, the detection limit being 1 mg/L (256). Only 25 μL of serum sample is required. More than 500 assays are possible and the enzyme is very stable if stored at 4 °C in air. Sensors that eliminate the interference of endogenous ammonia during the determination of creatinine in blood serum and urine samples are also available (257–259) The biocatalyst layer consists of a bienzyme system immobilized over an ammonia electrode catalyzing the following reactions:

$$NH_4^+ + \alpha\text{-Ketoglutarate} + NADH \overset{\text{GlDH}}{\rightleftharpoons} \text{Glutamate} + NAD^+ + H_2O \quad [31]$$

$$\text{Creatinine} + H_2O \xrightarrow{\text{Creatinine deiminase}} N\text{-Methylhydantoin} + NH_3 \quad [32]$$

Endogenous ammonia is converted to a nonresponsive product; creatinine does not react with glutamate dehydrogenase (GlDH) and is converted to ammonia when it reaches the immobilized creatinine deiminase.

6.5. Biological Purines

The determination of the purine-nucleotide metabolites xanthine, hypoxanthine, and inosine by biosensors is of special interest for the estimation of meat or fish freshness. After the death of a fish, adenosine triphosphate (ATP) in the fish tissue is quickly degraded to inosine monophosphate (IMP). Further enzymatic decomposition of IMP leads to the accumulation of hypoxanthine (Hx), which is used as an indicator of fish freshness. To quantify these compounds with biosensors, it is possible to perform amperometric measurements of the generated hydrogen peroxide or the consumed oxygen according to the following enzymatic reactions:

$$\text{Inosine Phosphate} \xrightarrow{\text{Nucleoside phosphorylase(NP)}} \text{Ribose-1-phosphate} + \text{Hypoxanthine} \quad [33]$$

$$\text{Hypoxanthine} + H_2O + \tfrac{1}{2}O_2 \xrightarrow{\text{XOD}} \text{Xanthine} + H_2O_2 \quad [34]$$

$$\text{Xanthine} + H_2O_2 + \tfrac{1}{2}O_2 \xrightarrow{\text{XOD}} \text{Uric acid} + H_2O_2 \quad [35]$$

A single-enzyme electrode (XOD) may be used to measure xanthine and hypoxanthine, while a two-enzyme electrode (XOD and NP) must be used to determine inosine.

A disposable hypoxanthine biosensor based on a microoxygen electrode monitors hypoxanthine in the concentration range 6.7–180 μM (260); xanthine and purine interfere. Xanthine oxidase (XOD) is also attached to preactivated commercially available membranes and successfully applied for hypoxanthine analysis in fish extract using a H_2O_2-sensitive electrode (261). Alternatively, XOD, immobilized via GA on a polycarbonate membrane, is mounted on the tip of a platinum electrode for detecting the generated hydrogen peroxide or the consumed oxygen (262, 263). A linear response of the sensor for hypoxanthine in the range 2.5–375 μM is observed (262). Inosine is also determined with the bi-enzyme configuration in which the FAD containing xanthine oxidase is immobilized onto the conducting organic electrode TTF/TCNQ, which catalyses the reoxidation of $FADH_2$ (264).

6.6 Cholesterol

Cholesterol can be determined by immobilizing the enzyme cholesterol oxidase (COD) in a layer over an oxygen electrode or a hydrogen peroxide electrode. Cholesterol oxidase is chemically immobilized onto nylon net (135, 265) or collagen membranes (267) and fixed onto an O_2 electrode:

$$\text{Cholesterol} + O_2 \xrightarrow{\text{COD}} \Delta\text{-4-Cholestenone-3} + H_2O_2 \qquad [36]$$

Several applications have been realized for the microdetermination of human bile cholesterol (265) and serum cholesterol (266, 267) using the bienzyme system COD–cholesterol esterase. COD is immobilized under mild conditions on collagen films; the H_2O_2 liberated is detected at a Pt electrode. The linear range of cholesterol determination is 0.1 μM to 8 mM, but the use of a nonenzymatic electrode is required to substract out interfering oxidizable species such as ascorbate, urate, and tyrosine (126, 268). A rapid (1-min) electrochemical assay for cholesterol in biological materials is also described. Ten microliters of sample is injected into a 50 °C thermostat cuvet containing soluble COD and cholesterol esterase (269). The enzyme COD and cholesterol ester hydrolase are immobilized in a modified 2-hydroxyethylmethacrylate gel, placed over a platinum electrode to detect H_2O_2. Free cholesterol in serum samples is measured as well as total cholesterol using this bi-enzyme sequence (270). An FIA manifold for cholesterol analysis is described and the influence of solution surfactants on electrode stability is discussed in Moody et al. (271).

6.7. Protein, Amino Acid, and Amine

D-Amino and L-amino acid-selective electrodes are based on the biocatalytic oxidation of amino acids:

$$\text{Amino acid} + H_2O + O_2 \xrightarrow{\text{AAO}} \text{R-CO-COOH} + NH_4^+ + H_2O_2 \qquad [37]$$

L-Amino acid enzymes immobilized on O_2 electrodes are used to determine methionine, leucine, phenylalanine, tyrosine, cysteine, lysine, and isoleucine (272). Kinetic measurements of H_2O_2 formation result in fast electrode responses (less than 12 s) using chemically bound L-amino acid oxidase (LAAO) covering a platinum electrode (170) to assay for cysteine, leucine, tyrosine, phenylalanine, tryptophane, and methionine. Potentiometric-selective electrodes for amino acids are constructed by immobilizing LAAO on chemically modified graphite (273). The interaction of hydrogen peroxide with the carbon surface groups appears to be the major contributor to the potentiometric response. Several amino acid electrodes monitoring the NH_4^+ ion have also been reported (68, 274–277).

Several L-glutamate-sensitive enzyme electrodes based on the activity of either glutamate dehydrogenase (GlDH), glutamate decarboxylase (GlDC), or glutamate oxidase (GlOD) have been reported (278 and references cited therein). Improved selectivity and sensitvity are obtained with the dehydrogenase enzyme (279, 280). The systems consist of GlDH coupled to redox mediators (3-α-naphtoyl-Nile blue (280) and Meldola blue (279)) adsorbed on graphite electrodes, leading to oxygen-dependent catalyzed NADH oxidation. Considerable enhancement in sensitivity is obtained using the GlDH-mediator–enzyme-mediator substrate-amplification technique:

$$\text{Glutamate} + \text{NAD}^+ + \text{H}_2\text{O} \overset{\text{GlDH}}{\rightleftharpoons} \alpha\text{-Ketoglutarate} + \text{NH}_4^+ + \text{NADH} \quad [38]$$

$$\alpha\text{-Ketoglutarate} + \text{Alanine} \overset{\text{GPT}}{\rightleftharpoons} \text{Glutamate} + \text{Pyruvate} \quad [39]$$

Where GPT is glutamate pyruvate transaminase. In equations 38 and 39, glutamate is recycled, so that small amounts of glutamate will produce a buildup of NADH in the reaction layer.

Recently, Guilbault et al. (282) demonstrated that superior glutamate sensors can be constructed by placing glutamate oxidase onto oxygen- and peroxide-based sensors. The probes have fast responses (less than 1 min), are specific for L-glutamate with no interference, and offer high sensitivity (1 μM). Microelectrodes for direct studies of glutamate in brain tissues are being constructed for in vivo studies.

Determining L-tyrosine in biological fluids is possible with sensors obtained by immobilizing apo-L-tyrosine decarboxylase over a CO_2-sensitive gas electrode (283). L-Lysine is determined in grains and foodstuffs with L-lysine decarboxylase mounted onto a CO_2 electrode (284). The electrode is stable for more than 40 days, exhibits a response time of 5–10 min, and has a linear range of L-lysine concentration of 50 μM to 0.1 M. It can also be monitored in a fermentor by using a L-lysine oxidase electrode operating in a continuous flow system. The enzyme is immobilized directly on the selective gas membrane of an O_2 sensor, by copolymerization with gelatin using GA; L-arginine, L-phenylalanine, and L-ornithine interfere in the analysis (285).

L-Lysine and L-arginine are determined in a rapid fashion by using a bi-enzyme-immobilized system, decarboxylase-diamine oxidase:

$$\text{R-CNNH}_2\text{COOH} \xrightarrow{\text{Decarboxylase}} \text{RCH}_2\text{NH}_2 + \text{CO}_2 \quad [40]$$

$$\text{RCH}_2\text{NH}_2 + \text{O}_2 + \text{H}_2\text{O} \xrightarrow{\text{Amine oxidase}} \text{RCHO} + \text{NH}_3 + \text{H}_2\text{O}_2 \quad [41]$$

The uptake of oxygen is linearly related to the substrate concentration in the 10 to 100 mM region (286).

Highly selective L-arginine biosensors are described where the ammonia liberated in the reaction sequence catalyzed by the enzymes arginase–urease is monitored potentiometrically. The probes exhibit linear responses in the range of arginine concentrations of 0.1 mM to 0.01 M (140) and 30 μM to 3 mM (287).

Several sensors for L-alanine are obtained by immobilizing L-alanine dehydrogenase over an ammonia gas-sensing electrode (288) or over an O_2 sensor (280). The enzyme catalyzes the specific deamination of alanine in the presence of the coenzyme NAD:

$$\text{L-Alanine} + \text{NAD}^+ + \text{H}_2\text{O} \rightleftharpoons \text{Pyruvate} + \text{NADH} + \text{NH}_4^+ \quad [42]$$

Co-immobilizing a second enzyme, NADH oxidase (280) or lactate dehydrogenase (288), permits the regeneration of NAD^+. Measurements may be completed in Tris-HCl or carbonate buffers, but borate and glycine buffers inhibit L-alanine dehydrogenase (289). Highly selective L-histidine electrodes are available (290, 291) to determine histidine in urine (291).

A potentiometric L-threonine selective sensor for determining L-threonine in biological fluids and foods utilizes threonine deaminase in conjunction with an NH_3 gas-sensing electrode. The biosensor exhibits a linear response to L-threonine concentration over the 0.1–200 mM range (292). Comparing L-tryptophan bacteria and immobilized enzyme electrodes shows that the enzyme probe is stable for less than 5 days but that the bacterial probe functions for approximately 3 weeks (293).

Assaying for total serum protein is possible by first hydrolyzing the protein in serum with pepsin and then determining tyrosine with a specific tyrosine electrode (294). The electrode is useful 15–20 days, with 10–20 assays per day. The limit of detection was 10 μM and the linearity extended up to 0.25 mM. Total protein in flow systems is determined by cleaving the protein in a reactor with protease, converting the amino acids to NH_3 by passage over glass-immobilized L-amino acid oxidase (LAAO). BSA was determined in the range of 0.1–100 μg/mL (295).

Glutamine is selectively determined using a glutamine-selective electrode. Comparing several biocatalysts (isolated enzyme, bacteria cells, kidney mitochondrial fractions) immobilized at an ammonia gas-sensing electrode shows

that tissue slices offer the most favorable combination of electrode properties (296). Quantification of glutamine in cerebrospinal fluid (297) and in a mammalian cell culture (298) is reported.

An electrode for the determination of L-aspartate is constructed by chemical immobilization of L-aspartase on an ammonia gas-sensing probe. The electrode response is linear in the concentration range 0.7–20 mM with a slope of −59 mV/decade. The biosensor is stable for more than 20 days (299).

6.8. Choline and Choline Ester

Enzyme electrodes for choline and acetylcholine (300, 301) and for the analysis of choline-containing phospholipids (303, 304) is obtained by immobilizing choline oxidase or choline oxidase and acetylcholinesterase on membranes at the tip of platinum electrodes. The formation of hydrogen peroxide is monitored:

$$\text{Choline} + 2O_2 + H_2O \longrightarrow \text{Betaine} + 2H_2O_2 \qquad [43]$$

The linear ranges generally achieved with such configurations are between 1 and 300 μM for choline and 1 and 600 μM for acetylcholine (300–302).

Choline-containing phospholipids are determined by co-immobilizing phospholipase D and choline oxidase onto a platinum electrode:

$$\text{Phosphatidyl choline} \xrightarrow{\text{Phospholipase D}} \text{Phosphatidic acid} + \text{Choline} \qquad [44]$$

Either the decrease of O_2 (303) or the formation of H_2O_2 is successfully monitored (304). The system has been applied to serum and amniotic fluids, with detection limits as low as 0.01 g/L (304).

6.9. Immunoelectrodes

The enzyme–antigen- or enzyme–antibody-linked enzyme immunoassay (EIA) has many advantages over radioimmunoassays (RIA), such as elimination of expensive counting equipment, elimination of radioactive waste, and cheap and stable reagents (305). The application of electrodes to monitor immunological reactions is described in the following sections.

6.9.1. ENZYME-LINKED ANTIBODIES

An enzyme immunoelectrode suitable for the assay of human serum albumin and insulin uses an oxygen electrode covered with an antibody-containing nylon net kept in place with an O-ring. From 1 to 25 ng/L of albumin and 5 to 100 ng/L of insulin can be assayed (306). A specific sensor for the tumor antigen α-fetoprotein (AFP) is prepared by immobilizing anti-AFP antibody covalently on a membrane prepared from cellulose triacetate, 1,8-diamino-4-aminomethyl octane, and GA (307). The sensor is applied to an EIA based on competitive Ab/

Ag reaction with catalase-labeled antigen. After competitive binding of free and catalase-labeled AFP, the sensor is examined for catalase activity by amperometric measurement after addition of H_2O_2. AFP can be assayed in the range 10–0.01 ng/mL.

Several assays for human chorionic gonadotropin (HCG) are based on the Ag/Ab reaction in conjunction with selective electrodes (308–311). The assay is monitored using a cyanogen bromide treated electrode covered with the corresponding antibody (311). The potential of the modified electrode shifts in the positive direction after contact with a solution of the antigen. The change in potential is approximately proportional to the HCG concentration. The technique applied to human urine samples shows a specific response to only HCG. Alternatively, immobilized acetylcholinesterase (AchE) and a pH electrode can be used for specific HCG determinations (308). The required separation of enzyme conjugate (AchE antigen) from the assay mixture is accomplished with an immobilized antibody. The antibody to HCG is fixed in a membrane form with a polyethylene net. The AchE antigen and the free antigen competitively react with the membrane. After completion of the reaction, the activity of the enzyme–antigen fixed on the membrane is measured with a pH electrode (glass) placed against the membrane in an acetylcholine solution. The pH variation is dependent on the enzyme activity in the test solution (the detection limit for HCG is 1 nM).

A "sandwich" assay for HCG using GOD as label is also described (309). The procedure is based on an amperometric enzyme immunoassay with an electrode-immobilized antibody. The antibody electrode (activated glassy carbon) is used both to separate the assay and to monitor the activity of the bound enzyme label. Two monoclonal antibodies directed against different antigenic sites are used:

$$\text{E-anti-HCG} + \text{Antigen} \longrightarrow \text{E-anti-HCG} - \text{Antigen} \qquad [45]$$

$$\text{E-anti-HCG} - \text{Antigen} + \text{anti-HCG}\!\!\equiv \rightarrow \text{E-anti-HCG} - \text{Antigen} - \text{anti-HCG}\!\!\equiv \qquad [46]$$

First the immobilized GOD–anti-HCG is allowed to react with the antigen (equation 45). In a second step the antibody electrode is utilized to capture the GOD–anti-HCG complex (equation 46). After electrode capture, the catalytic activity of the bound enzyme label is measured by cyclic voltammetry in the presence of a redox mediator. The electrode is regenerated by soaking in urea for 5 min to break the Ab/Ag bond. Sensitivity of the assay is 9 mIU HCG/mL in serum.

Several antibody electrodes are available for determining hepatitis-B surface antigen (HBsAg) in biological fluids (312–314). Horseradish peroxidase is linked to anti-HBsAg gamma-globulins and the resulting labeled antibody is immobilized on gelatin membranes. The antigen concentration was measured potentiometrically with an iodide-selective electrode modified by fixing the active membrane onto the iodide sensor (312, 314). The electrode is dipped in

HBsAg solution to extract the antigen, then dipped in a solution of anti-HBsAg labeled with HRP according to the sandwich principle (305). The amount of HRP is measured in a peroxide iodide solution according to the HRP-catalyzed reaction:

$$H_2O_2 + 2I^- + 2H^+ \xrightarrow{\text{HRP}} I_2 + 2H_2O_2 \qquad [47]$$

The detection limit for HBsAg is 0.1 μg/L (314).

Bovine serum albumin (BSA) and cyclic AMP (cAMP) are determined by a competitive binding enzyme immunoassay (315). With urease as label, an ammonia gas-sensing electrode is used to measure the amount of urease-labeled antigen bound to a double-antibody solid phase by continuously measuring the rate of ammonia produced from urea as substrate. The method yields accurate and sensitive assays for proteins (BSA less than 10 ng/mL) and antigens (cAMP less than 10 nM), with fairly good selectivity over cGMP, AMP, and GMP.

The potentiometric determination of estradiol-17β in solution provides an example of a solid-phase competitive immunoassay (316). The anti-estradiol-17β antibodies are immobilized on a pig skin gelatin membrane. After incubation with HRP-labeled steroid and estradiol, the membrane is mounted over an iodide-selective electrode to measure the enzymatic activity. The electrode potential is a function of antigen concentration at levels ranging from 57 pM to 9.2 nM.

Very low levels of digoxin are accurately determined in human plasma using a competitive heterogeneous EIA with electrochemical detection (detection limit 50 pg/mL) (317). Digoxin in the sample and alkaline phosphatase-labeled digoxin compete for solid-phase Ab coated on the walls of reagent tubes. The labeled digoxin bound to the solid-phase Ab is determined by incubation with the enzyme substrate (phenylphosphate) and the phenol is quantified by oxidation in a thin layer cell under flow-through conditions.

6.9.2. BOUND ANTIGENS

An enzyme immunoassay using adenosine deaminase as the enzyme label has been described (318). Potentiometric rate measurements were made with an ammonia gas-sensing electrode. The immunoassay system employed is based on competition between a model haptenic group, dinitrophenyl (DNP) covalently coupled to adenosine deaminase, and free DNP hapten for the available binding sites on the anti-DNP antibody molecules. The detection limit is 50 ng antibody.

An immuno sensor for determining specific proteins uses a "liquid" antigen containing cardiolipin, phosphatidyl choline, and cholesterol immobilized onto an acetyl cellulose membrane. The membrane-bound antigen retains immunological reactivity to Wasserman antibody. The asymmetrical potential is dependent on the concentration of the antibody (319, 320).

Immobilizing the syphilis antigen makes it possible to assay for syphilis antibody in blood serum (321). The contact potential between bound antigen and antibody is measured, with very low voltage change (1–3 mV).

Potentiometric digoxin antibody measurements with antigen–ionophore-based membrane electrodes are successfully applied to serum samples (322). The antigen (digoxin), corresponding to the antibody to be measured, is chemically coupled to an ionophore (*cis*-diaminobenzo-18-crown-16) to form an antigen–carrier conjugate. The conjugate is incorporated into a plastic support membrane and that membrane is mounted in the sensing tip of a conventional potentiometric membrane electrode (K^+-selective sensor). The potential change that occurs after addition of the appropriate antibody is proportional to its concentration. Interference is negligible and sensitivity is high due to the great affinity of digoxin antibodies for the drug digoxin. Further innovation in the potentiometric membrane electrode design is obtained by limiting the antigen–antibody reaction to the sensor tip (323, 324). The electrode contains an antigen coupled to an ionophore and the reaction of the antigen with its antibody modulates the ion carrier properties of the ionophore and produces a potential change. This configuration has a highly attractive feature: The entire immunoreaction occurs within the tip of the sensor (antibody conservation) and exhibits reversible antigen responses. It is also possible with this probe to distinguish between monoclonal and polyclonal antibodies for 2,4-DNP (dinitrophenol) (324).

6.10. Drugs

There is considerable interest in the design of biosensors for drugs of abuse (cocaine, heroin, morphine, etc.), as well as for prescription pharmaceutical compounds (see 267, 325, and 326 for reviews). Typically these sensors are of the liquid membrane type, containing an organic compound reactive to the drug. A cocaine sensor, for example, consists of a PVC membrane plasticized with dibutyl phtalate, and containing tetraphenylborate as the active substance. A strychnine sensor uses a liquid membrane electrode based on ion pairs of strychnine with picrolonate or tetrakis (3-methylpheny)borate.

Allen and Hill (327) proposed new enzyme electrodes for determining drugs such as lidocaine and theophylline, using fixed amounts of antibody, ferrocene-labeled drug, enzyme and substrate. In the absence of the drug, the ferrocene drug conjugate binds to the antibody and no reference drug conjugate is available to mediate the flavoprotein. In the presence of the drug, the ferrocene conjugate competes for antibody and the catalytic current increases at a rate proportional to the content of the drug in the sample.

A polyvinyl chloride membrane electrode for heroin based on an ion-pair complex with tetraphenylborate is described (328). The sensor shows a near-Nernstian response over the heroin concentration range 0.01 M to 0.1 mM, with good selectivity for heroin in the presence of a number of adulterants and base compounds present in illicit heroin powders. A cocaine sensor proposed by Zeng (329) uses a cocaine picrylaminate membrane. The calibration curve is linear in the range 0.01 M to 10 μM, with a coefficient of variation of less than

1.3%. The method is simple, fast, and sensitive, but no comment is made on selectivity.

Determining the hydrochlorides of cocaine, morphine, and pilocarpine in bulk and prescription drugs is also possible with a chloride-selective electrode (330). No selectivity is possible. A probe for molecular recognition of opiate receptors was described by Fujii et al. (331). Finally, gas-phase biosensors can also be used to assay for drugs. Guilbault and Luong (332) describe a gas-phase cocaine sensor. An anti-benzoyl ecgonine antibody mounted on a 9-MHz piezoelectric crystal shows good selectivity on reaction with cocaine antigens in air. The crystal possesses reactivity for 7 days with 84% sensitivity remaining, with a reduction of 36% activity in 13 days. The sensitivity is 50 Hz/ppb, but the sensor could not operate at relative humidities greater than 50%.

A modified enzyme immunobiosensor for microdetermination of digoxin in air consists of a water adsorbing porous membrane containing antibodies, enzyme-labeled antigen, substrates, and an enzyme indicator (e.g., glucose-6-phosphate, NAD^+, glucose-6-phosphate dehydrogenase-labeled digoxin and anti-digoxin) (333). It has high sensitivity and good selectivity.

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Enzyme-Labeled Probes for Nucleic Acid Hybridization

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1. INTRODUCTION

The power and utility of recombinant DNA methods, which include techniques of nucleic acid hybridization, are based on the ability to manipulate DNA in defined ways. These molecular tools are dependent on the commercial availability of a variety of well-characterized enzymes for DNA and RNA modification: restriction endonucleases which recognize and cleave specific DNA sequences to create DNA fragments of unique size, DNA and RNA polymerases,

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and polynucleotide kinase. The use of these enzymes with amplified DNA segments, produced in specially engineered plasmid and viral vectors, and nucleotide substrates of high specific radioactivity makes it possible to produce highly radioactive nucleic acid probes. Chemical methods for synthesis of oligonucleotides of specific sequence are also useful in the preparation of hybridization probes.

Radiolabeled DNA probes are used routinely in a variety of gene mapping methods. Such experiments rely on annealing of the probes to homologous DNA sequences in electrophoretically separated DNA fragments produced by restriction endonucleases. Hybridization analysis of genomic DNA using a DNA probe prepared by synthesis of a DNA complementary to a specific messenger RNA, and its subsequent amplification by DNA cloning, provides structural information on intervening sequences (introns) within a gene. Alternatively, if a genomic DNA probe is annealed to the messenger RNA encoded by it, introns can be mapped more precisely by an S1 nuclease analysis (see below). DNA probes can be also used to map the 5′ or 3′ ends of RNA molecules to determine transcriptional start and stop sites, as well as to examine post-transcriptional processing. Finally, DNA probes are also used to identify recombinant DNAs by colony and plaque hybridization, and in the analysis of restriction fragment length polymorphism.

This article presents a brief review of the properties of enzymes used in preparing DNA and RNA probes for hybridization. Included are reaction conditions typically employed and examples of types of experiments generally performed with different probes. More comprehensive protocols may be found in *Molecular Cloning: A Laboratory Manual* (1) and *Current Protocols in Molecular Biology* (2).

2. 5′-END LABELING OF DNA AND RNA

2.1. General Properties of T4-Encoded Polynucleotide Kinase

T4-encoded polynucleotide kinase transfers the γ-phosphate of a nucleoside 5′-triphosphate to 5′-hydroxyl termini of DNA or RNA molecules, producing a nucleoside 5′-diphosphate and a 5′-phosphoryl RNA or DNA (3–5). A similar enzyme has been identified from bacteriophage T2-infected cells. The T4 enzyme, which comprises four identical subunits of 33 kDa, is the most extensively characterized and the enzyme of choice in labeling DNA and RNA. The gene of T4 that encodes this enzyme, *pseT*, also encodes a 3′-phosphatase (6, 7). The 3′-phosphatase is active on deoxyribonucleotides and on DNA, but is inactive on ribonucleotides and RNA. A T4 mutant, *pseT*1, encodes a protein that possesses an active polynucleotide kinase but has greatly reduced 3′-phosphatase activity (7, 8). Currently, polynucleotide kinase is purified from strains that overproduce this enzyme. Because these strains are mutant in the 3′-phosphatase of the *pseT* gene, the enzyme is essentially free of the associated 3′-phosphatase activity.

2.2. Substrate Preference

Polynucleotide kinase phosphorylates both DNAs and RNAs of varying lengths and nucleoside 3′-phosphates. However, the rate of the reaction and affinity of the enzyme for these substrates is dependent on the nature of the 5′-terminal residue, and on the length of the oligonucleotide (5). Single-stranded DNA is a preferred substrate (10-fold higher rate) relative to termini at gaps in double-stranded DNA. Protruding 5′-hydroxyl termini of double-stranded DNA are preferred over those in blunt-ended DNA, which is used more effectively than DNA with recessed 5′-hydroxyl ends. Nevertheless, efficient phosphorylation of the poorer substrates can be achieved with sufficiently high levels of ATP. The 5′-hydroxyl termini of nicked DNA are phosphorylated only very inefficiently by polynucleotide kinase.

The K_m for ATP varies from 14 to 140 μM in the "forward reaction" as a function of DNA substrate (9, 10). Because commercially available [γ-^{32}P]ATP (1–10 μM) is not sufficiently concentrated for use near the K_m for the enzyme, most procedures include [γ-^{32}P]ATP at or greater than 1 μM. For the T2-encoded enzyme, other ribonucleotides are also effective phosphoryl donors with K_m values ranging from 14 to 33 μM, depending on ribonucleotide, differing by only a factor of 2. Polynucleotide kinase absolutely requires Mg^{2+}, exhibiting an optimum at 10 mM (4). Reagents that stimulate activity include polyanions such as spermidine and the reducing agents 2-mercaptoethanol and dithiothreitol, with optima at 10 and 5 mM, respectively (5). In the absence of a reducing agent, the activity of polynucleotide kinase is reduced about 50-fold. The optimal pH range is between 7.4 and 8.0 with maximal activity at pH 7.6. Phosphate and pyrophosphate are inhibitory: 50% inhibition is observed at 20 mM inorganic phosphate or 5 mM pyrophosphate. Ammonium ions at 7 mM inhibit polynucleotide kinase by 75%.

5′-end labeling of DNA by T4-encoded polynucleotide kinase is usually employed in DNA sequence analysis by the chemical method of Maxam and Gilbert (11), and in S1 nuclease analyses. It is also used for phosphorylation of chemically synthesized linkers, a required step prior to their ligation to DNA by either bacteriophage T4 or *Escherichia coli* DNA ligase. Because restriction endonucleases produce DNA termini with 5′-phosphoryl ends, enzymatic methods for removal of 5′-phosphate groups from DNA or RNA by either bacterial alkaline phosphatase or calf intestinal phosphatase must first be performed with DNA restriction fragments (1, 2). The phosphatases are commonly inactivated and removed by phenol or phenol:chloroform extraction followed by ethanol precipitation. Alternatively, the addition of inorganic phosphate to 1 mM can be used to inhibit alkaline phosphatase activity during subsequent phosphorylation by polynucleotide kinase (3).

An alternative to the two-stage end-labeling protocol involves a phosphate exchange reaction catalyzed by polynucleotide kinase. In the presence of excess amounts of ADP, polynucleotide kinase transfers a 5′-phosphoryl residue from DNA or RNA to ADP in a reversal of the phosphorylation reaction (5, 12). At pH

6.2, the K_m for ADP is 200 μM in reactions containing restriction fragments with 5′-phosphoryl termini. Notably, the optimal pH for the exchange reaction is 6.2 as compared to 7.6 for the forward reaction. In the presence of [γ-^{32}P]ATP, polynucleotide kinase then transfers the γ-phosphate from ATP to the 5′-hydroxyl termini produced in the exchange reaction, allowing the labeling of 5′-terminal phosphate residue. At pH 6.2, the K_m for ATP in the exchange reaction is 4 μM. For the exchange reaction to work well, [γ-^{32}P]ATP must be present in large excess relative to the substrate to be labeled. Other factors that influence the reaction are as indicated for the forward reaction.

The forward reaction may be accomplished in a reaction volume of 20 to 50 μL in 50 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 1.7 mM spermine, 5 mM dithiothreitol, 0.1 mM EDTA, the DNA to be radiolabeled, 1–10 uM [γ-^{32}P]ATP, and 10–20 units of T4 polynucleotide kinase. For labeling of DNA by the exchange reaction, 50 mM imidazole-HCl, pH 6.2, is substituted for 50 mM Tris-HCl, pH 7.6, and ADP is included at 300 μM. Incubations are usually at 37 °C for 15–30 min.

To measure the extent of DNA labeling by these and the other procedures described in this review, the labeled products are precipitated with acid and collected on glass-fiber filters. To do so, a portion of the reaction mixture is added to 1 mL of 10% trichloroacetic acid containing 0.1 M sodium pyrophosphate and incubated on ice for ⩾10 min. The precipitate is collected by filtration onto Whatman GF/C filters soaked in 1 M HCl containing 0.1 M sodium pyrophosphate. The filters are washed with 20–50 mL of the above solution and then with 5–10 mL of ethanol. Radioactivity is quantitated by liquid scintillation counting.

3. 3′-END LABELING OF DNA WITH DNA POLYMERASES

3.1. General Properties of DNA-Directed DNA Polymerases

All DNA polymerases synthesize DNA in the 5′ → 3′ direction, incorporating deoxynucleoside monophosphates into a growing chain using the energy derived by the hydrolysis of the deoxynucleoside triphosphate substrates (13). All DNA polymerases require a template primer. The template strand directs the synthesis of a complementary DNA strand, which is initiated by extension of the 3′-hydroxyl terminus of a primer RNA or DNA which is base-paired with the template strand. DNA polymerases commonly used in preparing radiolabeled DNA include *E. coli* DNA polymerase I(14), the large fragment of DNA polymerase I (also referred to as the Klenow fragment, derived by proteolysis or by molecular genetic manipulation to result in a truncated polypeptide encoded in the C-terminal portion of the *E. coli pol*A gene (15, 16)), the thermostable DNA polymerase from *Thermus aquaticus* (*Taq* DNA polymerase, (17)) and the DNA polymerases encoded by bacteriophages T4 (18) and T7 (19). Forms of T7 DNA polymerase altered by chemical (Sequenase, U.S. Biochemicals; (20)) or mole-

cular genetic methods (Sequenase 2.0, U.S. Biochemicals; (21)) are commercially available and are used when long DNA product strands are desired. DNA polymerase III holoenzyme, the replicative DNA polymerase of *E. coli* (22), is also commercially available, although infrequently used for these purposes.

3.2. 3′-End Labeling of DNA Utilizing the DNA Polymerase and 3′ → 5′ Exonuclease Functions of DNA Polymerase

Except for the altered forms of T7 DNA polymerase and *Taq* DNA polymerase (23), all of the DNA polymerases identified above contain a 3′ → 5′ exonuclease function that specifically degrades single-stranded DNA to produce 5′-nucleoside monophosphates. In vivo, the 3′ → 5′ exonuclease functions during DNA synthesis to hydrolyze misincorporated nucleotides at the 3′-terminus of a growing DNA chain, and in doing so contributes as much as a factor of 100 to the overall fidelity of DNA replication (24, 25).

The 3′- ends of double-stranded DNAs can be labeled in vitro by a method involving both the DNA polymerase and the 3′ → 5′ exonuclease activities of various DNA polymerases (26). The method relies on the fact that under most conditions, the DNA polymerase function is more active than its associated 3′ → 5′ exonuclease. Given a double-stranded DNA with either protruding 3′-single-stranded termini or with blunt ends, these DNA polymerases will catalyze exonucleolytic degradation in the 3′ → 5′ direction, hydrolyzing the single-stranded DNA regions and invading the double-stranded region and releasing nucleoside monophosphates. In the presence of a sufficient concentration of a single α-^{32}P-labeled deoxyribonucleoside triphosphate, replacement of the corresponding nucleoside monophosphate that was removed by exonucleolytic hydrolysis from the double-stranded region of the DNA by the 3′ → 5′ exonuclease will be accomplished by nucleotide polymerization. This enzymatic reaction is a dynamic process because it results in cycles of removal and replacement of 3′-terminal nucleotides. The bacteriophage-encoded DNA polymerases exhibit a higher 3′ → 5′ exonuclease versus DNA polymerase activity ratio than *E. coli* DNA polymerase I, and are therefore the enzymes of choice for this end-labeling strategy.

The replacement synthesis method requires the use of a radioactive deoxynucleoside triphosphate specified by the DNA template. With a DNA of known sequence, the extent of degradation followed by replacement synthesis can be predicted. For example, DNA fragments with the blunt and recessed 3′-termini shown in Fig. 1*A* and *B*, respectively, may be specifically radiolabeled by replacement of the 3′-terminal dCMP residue with its radioactive counterpart derived from [α-^{32}P]dCTP. Likewise, with a DNA fragment of known sequence with a 3′ protruding end, the method will allow the degradation of the single-stranded DNA segment, and, in the presence of all four deoxyribonucleoside triphosphates, labeling of the 3′-terminal nucleotide in the resulting double-stranded DNA molecule.

These procedures may be adapted to allow incorporation of a nucleotide analog at a specific site in the 3′-end of a DNA. DNAs end-labeled by the general

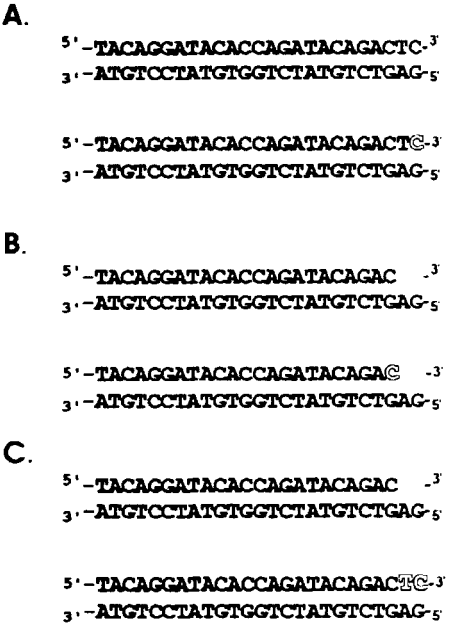

Fig. 1. Substrates for 3′-end labeling with DNA polymerase. (*A*) *Top:* unlabeled blunt-ended DNA; *bottom:* blunt-ended DNA specifically radiolabeled at its 3′ end. (*B*) *Top:* unlabeled DNA with a recessed 3′ end; *bottom:* the same DNA specifically radiolabeled at its 3′ end. (*C*) *Top:* unlabeled DNA with a recessed 3′ end; *bottom:* the same DNA converted to a blunt-ended form, specifically labeled at its 3′ end.

method are useful in S1 nuclease mapping of primary transcripts, as substrates for DNA sequencing reactions using the chemical method of Maxam and Gilbert and as molecular weight markers for gel electrophoresis.

3.3. 3′-End Filling Utilizing DNA Polymerase to Generate Blunt-ended DNAs

While the replacement DNA synthesis method for 3′-end labeling requires both the 3′ → 5′ exonuclease and DNA polymerase functions of DNA polymerases, the activity of the 5′ → 3′ DNA polymerase alone is sufficient to produce radiolabeled blunt-ended DNA fragments with substrates containing protruding 5′ termini. For example, the recessed 3′ terminus shown in Fig. 1*C* can be extended by DNA polymerase in the presence of dTTP and dCTP to yield the blunt-ended DNA fragment indicated.

In the absence of deoxyribonucleoside triphosphates, DNA polymerases with associated 3′ → 5′ exonucleases would eventually degrade duplex DNA to mononucleotides. The replacement DNA synthesis method thus requires sufficiently high levels of deoxyribonucleotides to maximize nucleotide poly-

merization relative to nucleotide excision. Reaction mixtures include 65 mM Tris–HCl, pH 8.0, 6 mM $MgCl_2$, 6 mM mercaptoethanol, and 50 μM deoxyribonucleotides, the composition of which is determined by the nucleotide sequence of the termini and by the chosen labeling strategy. DNA polymerase is used at a molar ratio near 1.0 relative to primer termini in the DNA substrate. Incubation is at 11 °C for 0.25–1 h. Strategies requiring exonucleolytic degradation of ⩾12 nucleotides will require longer incubations; incubation at temperatures higher than 11 °C can promote degradation beyond the point where a blunt-ended DNA is generated as a result of partial denaturation of the duplex DNA and subsequent hydrolysis of the termini by the 3′ → 5′ exonuclease. Reaction conditions for end-filling of 3′ recessed ends are similar to those described above except that the large fragment of DNA polymerase I (1 unit) is the preferred enzyme and incubations are carried out at 20 °C for 10–20 min.

The radiolabeled DNA may be separated from unincorporated radioactive nucleotides by conventional gel filtration using Biogel P-100 or Sephadex G-100, or by spin dialysis in buffer containing 10 mM Tris–HCl, pH 8.0, and 1 mM EDTA. In either case, the labeled DNA is recovered in the excluded volume.

3.4. 3′-End Labeling with Terminal Deoxynucleotidyl Transferase

Terminal deoxynucleotidyl transferase (TdT) polymerizes DNA by the same mechanism as DNA polymerases but lacks a requirement for a template DNA strand to direct synthesis (27, 28). TdT prefers single-stranded DNA or duplex DNA containing 3′ protruding ends, and is often used in the synthesis of a homopolymeric tail. Nucleotide incorporation is influenced by the divalent cation in the reaction: Purine nucleotides are incorporated preferentially in the presence of Mg^{2+}, while incorporation of pyrimidine nucleotides is favored in the presence of Co^{2+}. The use of nucleotide analogs (dideoxynucleotides or cordycepin triphosphate) results in the incorporation of a single nucleotide (29).

3′-End labeling with TdT is accomplished by incubation at 37 °C for 0.5–1 h in a reaction mixture (10–50 μL) containing 0.1 M sodium cacodylate, pH 7.0, 10 mM of Mg^{2+} or Co^{2+}, 1 mM DTT, 0.5 mg/mL BSA, DNA (4 pmol in 3′ termini), and 10 units of TdT. DNA synthesis can be measured and the product purified by gel filtration, as described earlier.

4. UNIFORM LABELING OF DOUBLE-STRANDED DNA

4.1. Uniform Labeling by Nick Translation

Among the DNA polymerases studied to date, *E. coli* DNA polymerase I and *Taq* DNA polymerase are unusual because they possess a 5′ → 3′ exonuclease, which specifically degrades double-stranded DNA, or RNA annealed to single-stranded DNA. In vivo, the 5′ → 3′ exonuclease of DNA polymerase I functions during bacterial DNA replication to hydrolyze RNA primers involved in priming of DNA synthesis, and during DNA repair to hydrolyze short regions of DNA

containing modified nucleotides. The products of digestion are mononucleotides and oligonucleotides 10–20 residues long.

Using double-stranded DNA with one or more single-strand discontinuities (nicks or breaks in the phosphodiester backbone), DNA polymerase I can extend the available 3′-OH termini in the presence of the four deoxyribonucleoside triphosphates. The reaction requires the concerted action of both the 5′ → 3′ DNA polymerase and 5′ → 3′ exonuclease functions of the enzyme. Nucleotide hydrolysis in the 5′ → 3′ direction concomitant with nucleotide polymerization results in translocation of the position of the discontinuity by a process termed "nick translation" (Fig. 2) (30). Discontinuities or nicks in DNA can be introduced into intact DNA by limited digestion with pancreatic DNase I, which generates 3′-hydroxyl termini in double-stranded DNA. If radioactive nucleotides are used in the reaction with DNA polymerase I, randomly and uniformly labeled DNA is produced (31).

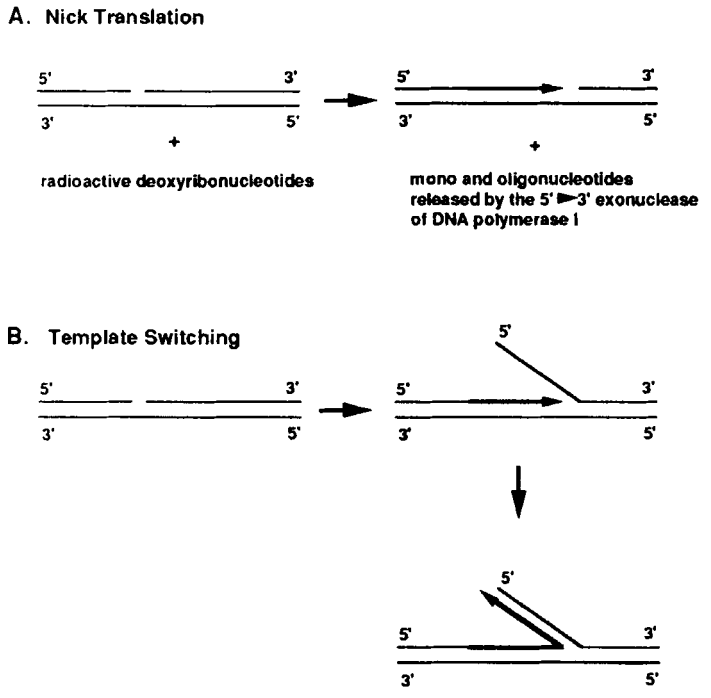

Fig. 2. Strategies for uniform labeling of double-stranded DNA. (*A*) Nick translation involves the 5′ → 3′ exonuclease and DNA polymerase functions of *E. coli* DNA polymerase I in the translocation of a single-strand break in a DNA strand. Translocation of the breakpoint occurs in the 5′ → 3′ direction as a result of concomitant nucleotide hydrolysis and polymerization. (*B*) Template switching involves the extension of a DNA chain at a single-strand break, in a reaction where DNA is duplicated, rather than replaced as in nick translation.

Highly radiolabeled DNA (0.1–0.3 μg, 10^8 cpm/μg) is produced by nick translation at or below 20 °C in the presence of DNase I(20 pg), in buffer containing 50 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol, 10% glycerol, three nonradioactive deoxyribonucleotides at 20–50 μM each, 0.1 mCi of the fourth deoxynucleotide (α-^{32}P-labeled, 3000 Ci/mmol), and 1–10 units of DNA polymerase I. This method relies on the combined activities of DNase I and DNA polymerase I to generate, and "translate" nicks, respectively (31). Because DNase I is labile at low protein concentrations, immediate use after dilution from a more concentrated stock is recommended. Addition of excess DNase I or prolonged incubation results in degradation of the resulting radioactive DNA to oligonucleotides. At temperatures greater than 20 °C, localized denaturation at the positions of the discontinuities in the double-stranded DNA may occur, resulting in template-strand switching during nick translation. This reaction generates forked structures in regions where a DNA strand is duplicated rather than replaced (Fig. 2).

To monitor the progress of the reaction, aliquots of the reaction mixture are removed at intervals of ~20 min and acid-insoluble radioactivity is measured as described previously. When the level of acid-precipitable radioactivity reaches a plateau, the master reaction is terminated by addition of EDTA to 20 mM on ice. The radioactive DNA can be isolated by gel filtration as described previously, with or without prior removal of the enzymes by phenol extraction.

4.2. Uniform Labeling with Oligonucleotide Primers

An alternative method for preparing uniformly labeled DNA is by oligonucleotide-primed DNA synthesis with hexanucleotides (or longer oligomers) of random sequence (30). Oligonucleotides of random sequence will anneal to a variety of homologous locations on a single-stranded DNA. Once annealed, they serve as primers for DNA synthesis by DNA polymerases. The method can also be used with supercoiled plasmid DNAs and linear duplex DNAs, including restriction fragments, and for preparation of radioactive complementary DNA (cDNA) from a mRNA population by reverse transcriptase. The highly radioactive DNA that is produced (~10^9 cpm/μg) is generally about 800 nt in length, and the reaction consumes 80–90% of the radioactive nucleotide used.

The large fragment of DNA polymerase I (Klenow fragment, 76 kDa), lacks the N-terminal amino acids of the intact enzyme, and is the preferred enzyme for this method because it lacks the 5′ → 3′ exonuclease function, which has the potential to degrade annealed primers. The Klenow fragment is also useful for 3′-end labeling of either recessed or protruding 3′ termini as described earlier, in synthesis of the second strand of complementary DNA in cDNA cloning, and in in vitro mutagenesis involving conversion of single-stranded DNA templates to their double-stranded form. In addition, the Klenow fragment is used in DNA sequencing by the chain-termination method of Sanger, which involves extension of a specific primer annealed to single-stranded DNA.

The DNA to be radiolabeled (0.1–0.3 μg) is mixed with a molar excess of random oligonucleotides and denatured in a boiling water bath for 1–3 min, and

then immediately placed on ice (32). Denaturation is usually carried out in a small volume (less than 5 μL) in a sealed glass capillary or in a tube overlaid with mineral oil to minimize evaporation. The denatured DNA is added to a reaction mixture (25 μL total volume) containing 0.1 M HEPES-KOH, pH 6.6,10 mM $MgCl_2$, 0.1 mM each of dATP, dGTP, and dTTP, 50 μCi [α-^{32}P]dCTP (3000 Ci/mmol), 0.5 mg/mL nuclease-free bovine serum albumin, and 2.5 units of the large fragment of DNA polymerase I. The intact form of DNA polymerase I can be used but results in DNA of lower specific radioactivity due to degradation of annealed primers and of the DNA products by its associated 5′ → 3′ exonuclease. With either enzyme, a pH of 6.6 appears to improve labeling efficiency, presumably because the 3 → 5′ exonuclease is relatively less active than at higher pH. Incubation is performed at 15 °C for 3 h to overnight. Labeling efficiency may be monitored and the radioactive product isolated as described earlier.

5. STRAND-SPECIFIC PROBES

5.1. Production of Strand-Specific DNA Probes

Given a recombinant of bacteriophage M13, strand-specific DNA probes can be prepared either by extension of an oligonucleotide primer annealed to the single-stranded viral DNA in a region upstream from the site of insertion, or by extension of random oligonucleotides. Derivatives of M13 such as M13mp18 and M13mp19 (33), which were constructed primarily for enzymatic sequencing of DNA, contain a region complementary to commercially available oligonucleotides, termed "universal primers." In the presence of radioactive deoxyribonucleotides, extension of the annealed primer by either the large fragment of DNA polymerase I, T4 DNA polymerase, or T7 DNA polymerase (modified or unmodified), results in the production of strand-specific DNA probes to the region inserted into these vectors. Reaction conditions for production of strand-specific DNA probes using the large fragment of DNA polymerase I have been described (34). The radiolabeled products are generally short because the enzyme has a low processivity, incorporating 10–20 nucleotides per primer binding event. By contrast, the T7-encoded enzyme does not dissociate from the primer terminus until it has synthesized a complete complementary strand of the viral DNA template. Thus, when long radiolabeled DNA strands are desired, T7 DNA polymerase is the preferred enzyme. Either method requires the subsequent purification of the labeled DNA product away from the unlabeled DNA template, usually by restriction endonuclease digestion and gel electrophoresis under denaturing conditions (1, 2).

Strand-specific DNA probes of high specific radioactivity are prepared by first annealing a primer to the single-stranded M13 DNA and then extending it with DNA polymerase (34). Annealing is accomplished in the presence of 100-fold molar excess of primer to DNA template (0.02–2 μg) in a reaction mixture containing 50 mM NaCl, 10 mM Tris-HCl, pH 7.5, and 7 mM $MgCl_2$. The mixture is

incubated for a few minutes in a water bath at 90°C, and then the water bath is allowed to cool to room temperature. Extension of the annealed primer is accomplished by addition of dithiothreitol to 2 mM, dGTP, dCTP, and dTTP to 300 μM, 50–100 μCi [α-^{32}P]dATP, and 1–2 units of the large fragment of DNA polymerase I. Following incubation at 20°C for 0.5 h, nonradioactive dATP is added to increase its concentration to 300 μM, and incubation is continued for 15–30 min to allow further extension of the radioactive DNA. The enzyme is inactivated by incubation at 70°C for 5 min, and the reaction is placed on ice. At this point, the amount of DNA synthesis can be measured by acid precipitation and liquid scintillation counting. The reaction buffer is then adjusted appropriately depending on the restriction enzyme(s) to be used to cleave the DNA for subsequent purification of the radiolabeled DNA strand by electrophoresis under denaturing conditions.

Under the above conditions, the radioactive dATP present in the first stage of DNA synthesis is the limiting component of the reaction. Depending on the amount of template DNA present, primers may be extended for only a short length. The addition of nonradioactive dATP during the second stage of extension minimizes this problem.

5.2. Production of Strand-Specific RNA Probes

For some applications, RNA probes are desirable because RNA–DNA hybrids are thermodynamically more stable than DNA–DNA hybrids. In addition, while the method described for production of strand-specific DNA probes requires purification of the radiolabeled DNA away from the unlabeled template, radioactive RNA probes may be obtained simply by treatment of the reaction mixture with RNase-free DNase I following RNA synthesis.

Several vectors have been developed for the synthesis of strand-specific RNA probes. These vectors contain transcriptional promoters for bacteriophage SP6, T3- and T7-encoded RNA polymerase, and sites for insertion of foreign DNA downstream from these promoters (35–38). Some of these vectors have been designed to express one strand or the other of target genes by placement of the phage promoters in orientations that flank and read into the insert. RNA can be produced by use of an in vitro transcription system containing the appropriate phage-encoded RNA polymerase, the recombinant DNA, and ribonucleoside triphosphates.

RNAs produced in vitro are useful in the development of in vitro splicing systems where the RNA produced is a primary transcript. In addition, the RNA can be used as a template for in vitro translation, and in mapping exons and introns in genomic DNA. Genes under control of bacteriophage promoters can also be expressed in bacterial cells to facilitate expression and/or purification of the proteins they encode.

In vitro synthesis of RNA with phage-encoded RNA polymerases is performed in a reaction mixture (10–50 μL) containing 40 mM Tris-HCl, pH 7.9, 6 mM $MgCl_2$, 2 mM spermidine-HCl, 1 mM dithiothreitol, 0.4 mM each of ATP, CTP, and UTP, 10–50 μCi [α-^{32}P]GTP, 20 mM of linearized plasmid DNA, 1 unit

of RNase Block II (an RNase inhibitor, Stratagene), and 1 unit of phage-encoded RNA polymerase. Incubation is at 37°C for 10 min. RNA synthesis can be measured by acid precipitation. The radioactive RNA is isolated by incubation of the reaction mixture with 1 μg of RNase-free DNase I for 15 min at 37°C, followed by extraction with phenol:chloroform (1:1) and ethanol precipitation to deproteinate the sample and remove most of the unincorporated ribonucleotides. The sample is then suitable for use directly as a hybridization probe.

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DNA Restriction Enzymes and RFLPs in Medicine

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1. RESTRICTION ENZYMES (ENDONUCLEASES)

1.1. General Information

Restriction enzymes are a revolutionary class of proteins discovered in prokaryotic organisms in the early 1970s (1-5). These enzymes reproducibly cut within double-stranded DNA molecules at specific nucleotide recognition sequences, termed restriction sites, hence their more specific appellation as restriction endonucleases (Fig. 1). Their complete range of enzymatic functions is not fully

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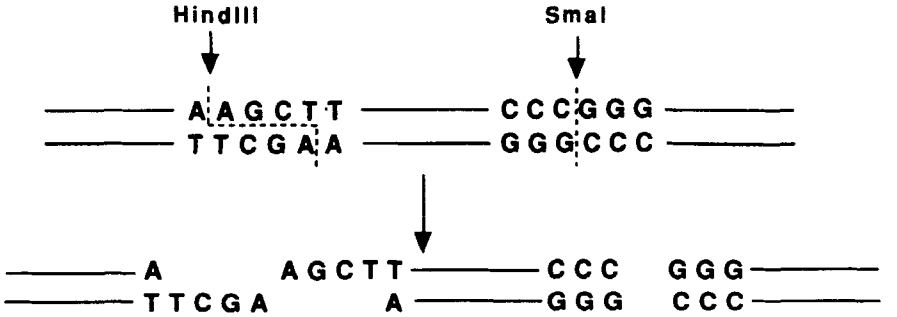


Fig. 1. Restriction endonuclease action. A region of DNA containing the 6 nucleotide recognition sequences for the class II restriction enzymes *Hin*dIII and *Sma*I is shown. *Hin*dIII cuts both strands of the DNA molecule at the recognition site asymmetrically, producing single-stranded "sticky ends." These unpaired regions are termed "sticky" because of their ability to easily pair with other DNA fragments cut by the same enzyme, thus forming hybrid molecules, which become the essential materials for cloning molecular probes. *Sma*I cuts the recognition sequence symmetrically to produce "blunt ends," which are less specific but still useful for cloning experiments.

understood, but restriction enzymes appear to play a major protective role in bacteria by destroying DNA of infecting bacteriophage viruses. Bacteria have evolved an ingenious system of modification methyltransferases to protect their own DNA, which may also contain recognition sequences, from restriction enzymes. The modification enzymes add a methyl group within the recognition sequence, thereby preventing digestion by the endonuclease at that site.

Restriction endonucleases are designated using three italicized letters to describe the genus and species of bacterium from which it was isolated followed by a roman numeral referring to its place in the order of restriction enzymes isolated from that organism. For example, *Kpn*I is the first enzyme isolated from *Klebsiella pneumoniae* and *Pvu*II is the second enzyme from *Proteus vulgaris*. Additional identifying information as to substrains or special features is added as letters or arabic numerals between the initial three letter descriptor and the final roman numeral (e.g., *Eco*RI, *Bam*HI, *Hind*III).

These enzymes are extraordinarily abundant; over 1200 restriction endonucleases had been isolated and characterized by early 1990. Of three classes defined, type II restriction enzymes, which generally cut within their recognition sequences, have found uses in a host of biomedical research and diagnostic applications to be discussed below. Type I enzymes cut nonspecifically many nucleotides distal to specific recognition sequences and contain both restriction enzyme and DNA modification (see below) activities on different subunits of multienzyme complexes. Type III restriction enzymes share the multienzyme aspects of type I enzymes but vary in other properties such as ATPase activity and cofactor requirements.

Type II restriction enzymes (Table 1) require physiologic pH, usually divalent cations (primarily Mg^{2+}), and, most importantly, a DNA substrate with the

proper recognition sequence. Recognition sequences generally consist of 4–6 nucleotides. The theoretical frequency of encountering a recognition sequence on a DNA molecule can be expressed by 4^n, where n is the length of a precise recognition sequence. Thus, *Eco*RI, with the 6 base recognition sequence 5′-GAATTC-3′, would be expected to cut DNA once every 4096 nucleotides. Enzymes with longer recognition sequences of 8 nucleotides or more, such as *Not*I, are particularly useful for DNA mapping projects, particularly when used in concert with more frequent cutters. Recognition sequences generally are palindromic, that is, they read the same 5′ to 3′ on each DNA strand (e.g., 5′-GAATTC-3′ is 3′-CTTAAG-5′ on the opposite strand).

1.2. Restriction Enzymes and Molecular Cloning

Depending on the pattern of digestion within the recognition site, restriction enzymes produce DNA restriction fragments that are either flush at each end

TABLE 1

Some Commonly Used Type II Restriction Endonucleases

Name	Microorganism	Recognition Sequence and Cleavage Pattern	*Number of Sites*[a]			
			λ	ΦX174	pBR322	M13mp7
*Ava*I	*Anabaena variabilis*	C↓(Py)CG(Pu)G	8	1	1	1
*Bam*HI	*Bacillus amyloliquefaciens* H	G↓GATCC	5	0	11	2
*Bgl*I	*Bacillus globigii*	GCC(N)$_4$↓NGGC	29	0	3	1
*Bgl*II	*Bacillus globigii*	A↓GATCT	6	0	0	1
*Dde*I	*Desulfovibrio desulfuricans*[b]	C↓TNAG	104	14	8	29
*Eco*RI	*Escherichia coli* RY13	G↓AATTC	5	0	1	2
*Hind*III	*Haemophilus influenzae* R_d	A↓AGCTT	6	0	1	0
*Hpa*II	*Haemophilus parainfluenzae*	C↓CGG	328	5	26	19
*Kpn*I	*Klebsiella pneumoniae* OK8	GGTAC↓C	2	0	0	0
*Mbo*I	*Moraxella bovis*	↓GATC	116	0	22	8
*Not*I	*Nocardia otitidis-caviarum*	GC↓GGCCGC	0	0	0	0
*Pvu*I	*Proteus vulgaris*	CGAT↓CG	3	0	1	1
*Sma*I	*Serratia marcescens* S_b	CCC↓GGG	3	0	0	0
*Taq*I	*Thermus aquaticus* YTI	T↓CGA	121	10	7	14

Note: N, any nucleotide; Py, pyrimidine; Pu, purine; ↓, site of single-strand cleavage (mirror image on complementary strand).

[a] Number of cleavage sites in bacteriophages λ and ΦX174, and plasmids pBR322 and M13mp7.
[b] Norway strain.

(blunt-ended) or have short, single-stranded, overhanging or "sticky" ends (Fig. 1). Because of the palindromic nature of recognition sequences, restriction fragments with overhanging ends readily anneal with one another, facilitating the insertion of other pieces of DNA into a circular bacterial plasmid which has been linearized at a single restriction site. Following plasmid transfection into bacteria and growth overnight, this process of creating and propagating recombinant DNA molecules is the paradigm for "cloning" a discrete nucleic acid segment. So convenient for experimental manipulations are overhanging ends that investigators frequently may ligate short oligonucleotides incorporating restriction enzyme recognition sequences to naturally or experimentally derived flush-ended DNA molecules. Following digestion with the appropriate enzyme, these DNA species may then be easily incorporated into recombinant constructs.

1.3. Restriction Enzymes, Chromosomal Mapping, and Gene Expression

In addition to creation and manipulation of recombinant DNA molecules, another major research use of restriction enzymes is the analysis of unkown pieces of DNA including newly identified cDNA or genomic DNA clones. Using known restriction enzyme sites within DNA of cloning vectors as anchors, it is possible to digest unknown DNA fragments with one or two (double digest) restriction enzymes to generate an ordered map of restriction sites. Such maps allow investigators to identify areas between unknown fragments and to target specific regions for nucleotide sequencing reactions. Restriction enzymes are also used indirectly to target regions in unmapped DNA fragments that may yield expressed genes; nucleotide sequences immediately upstream of many genes contain nonmethylated CG-rich regions that may be identified by such enzymes as *Hpa*II (7, 8).

Technical Notes. While not always essential, it is desirable to prepare relatively pure DNA for restriction digestion studies, since the presence of contaminating proteins, salt, ethanol, or chelators can markedly affect results. While recognition and cleavage of DNA is generally quite precise, inappropriate digestion at other sequences, referred to as "star activity," may occur if reaction conditions such as pH or salt deviate significantly from manufacturers' recommendations or in the presence of organic reagents. Most restriction enzymes are stored in glycerol to prevent freezing during storage at −20 °C, and star activity has also been reported if glycerol exceeds 10% in the reaction mixture.

A unit of restriction enzyme activity is the amount that will completely cleave 1 μg of a control DNA (usually bacteriophage λ) into its constituent restriction fragments in 1 h. Not all DNAs will necessarily be similarly processed, and it is conventional to add a two- to threefold excess of a restriction enzyme per microgram of DNA in the reaction. Most restriction enzymes are maximally active at 37 °C. The optimal temperatures for several are 58–65 °C; these reactions are overlaid by a drop of mineral oil to minimize evaporation. Rarely, optimal results are obtained below 37 °C. Stability of enzymes varies greatly. A number

retain virtually complete activity over many hours; others show significant losses within 1 h. Depending on the enzyme used, digestion intervals are usually 1–3 h. Prolonged digestion has potential risks if small amounts of contaminating nucleases copurify with the DNA to be analyzed.

2. RESTRICTION FRAGMENT-LENGTH POLYMORPHISMS

Apart from their utility as vehicles to facilitate molecular cloning during appropriate phases of research into the molecular basis of many diseases, restriction enzymes provide a single extraordinarily powerful tool for studying a number of biomedical problems in genetics, neoplasia, and infectious diseases. This tool is the restriction fragment-length polymorphism or *RFLP*.

2.1. RFLPs Defined

Nucleotide changes arise constantly at a low level in the DNA of humans as well as other organisms and are transmitted to offspring. Sometimes these changes have dire phenotypic consequences and are termed mutations. Hemoglobin S, which causes sickle cell disease, results from a single nucleotide change that changes a single amino acid. Generally, nucleotide changes are harmless and do not affect gene products. Many occur in the intervening sequences between coding portions of human genes. When nucleotide changes abolish or create a new recognition sequence for a restriction enzyme, digestion of the sample with that enzyme produces larger or smaller fragments (Fig. 2). Different sized fragments detected with a probe (see Technical Notes below) are termed polymorphic, and since they are detected via the use of restriction endonucleases, they have been termed restriction fragment-length polymorphisms (RFLPs).

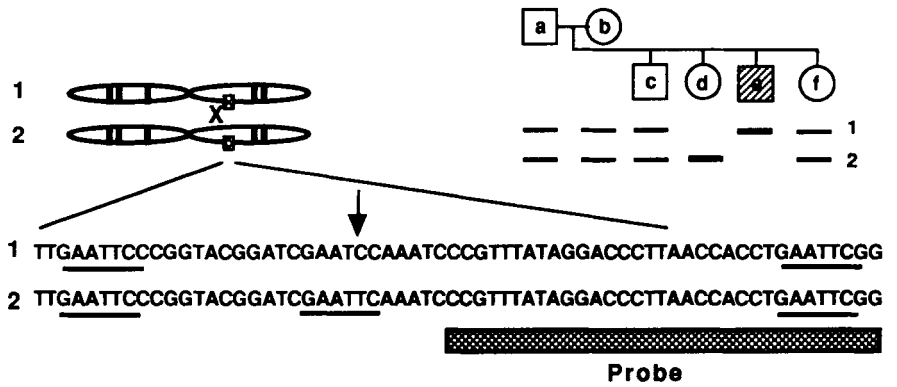


Fig. 2. Restriction fragment length polymorphism. See text for explanation and details.

2.2. RFLPs and Alleles in Human Genetic Disorders

The central importance of RFLP markers in modern hunts for disease-causing genes and the application of this knowledge to families at risk for the disease can be seen in Table 2, which lists the sequence of events followed from the identification of a disorder as genetic in origin to the ultimate characterization of the gene responsible for the disease. Note the importance of RFLPs at every step, initially chromosomal localization, then sublocalization to provide a jumping-off point for molecular approaches to the gene itself, and finally the use of intragenic RFLP markers to follow disease-bearing chromosome or detect specific mutant genes within families.

RFLPs are exceptionally powerful tools for tracking genetic disorders because they allow identification of individual chromosomes. Figure 2 shows a hypothetical region from an autosomal pair. One chromosome (1), when cut with the restriction endonuclease *Eco*R1 (recognition sequence GAATTC) gives a single hybridizing fragment on Southern blots. The DNA sequence on the second chromosome (2) contains a single nucleotide change which creates an extra *Eco*R1 site, thus the DNA piece that hybridizes with the probe is smaller than from chromosome 1. The identifiable differences in this region of the chromosome are sometimes referred to as *alleles.* Each parent (a and b) is heterozygous (or *informative*) for this RFLP, having one copy of allele 1 and one copy of allele 2.

2.2.1. LINKAGE OF RFLPs TO GENETIC DISEASES

Informative RFLP markers permit the tracking of chromosomes in prior and subsequent generations. Note in Fig. 2 that each child has inherited an RFLP allele from each parent; where identical-sized alleles have been inherited, there is usually an increased intensity of hybridization reflecting the difference in allele dosage.

In the search for genes causing single gene disorders, investigators generally begin with the inheritance pattern and test a large number of RFLP markers

TABLE 2

Sequence of Events in the Molecular Pathogenesis of Genetic Disorders

Stage	Geographical Knowledge of the Genetic Disorder
Initial	Inheritance defined only as a single gene disorder; autosomal (dominant, recessive); sex-linked
Early	Chromosomal localization by RFLP analysis
Intermediate	Sublocalization by closely linked and flanking RFLP markers; reliable genetic counseling and prenatal diagnosis
Late	Chromosome "walking" and "hopping" from RFLPs; search for conserved and expressed gene sequences
Final	Identification and characterization of the disease gene, along with specific disease mutations; detection of mutations or risk assessment using intragenic RFLPs; definitive genetic counseling and prenatal diagnosis

assigned to specific chromosomes, seeking a pattern of inheritance with a single marker to localize the disease to a given chromosome. The tests are most useful with large family pedigrees containing many diseased individuals or using many families with affected persons. A likelihood (or LOD) score of >1000:1 indicating that association is not random generally triggers further analysis using more RFLP probes from other regions of that chromosome. Higher LOD scores indicate closer proximity to the disease locus, since the likelihood an RFLP marker is generally proportional to its distance from a point. This phenomenon is termed *linkage.* Multipoint linkage analysis using RFLP markers associated with a particular disease can indicate the position of markers relative to the disease and one another and thus define markers on both sides of a disease locus. Figure 3 provides a map of RFLP markers linked to the neurofibromatosis-1 locus on chromosome 17. The closest linked flanking markers are generally chosen as sites from which detailed physical mapping and cloning strategies aimed at the isolation of the disease gene are launched.

If a mutant gene responsible for a recessive disease X was known to be close to the *Eco*R1 RFLP in Fig. 2, and son e was affected with the disease, one could infer that the mutation resides on the chromosome bearing allele 1 in each carrier parent. Similarly, we could say son c and daughter f are both likely to carry one chromosome bearing the mutation (we cannot say from which parent they inherited it using this RFLP). We could also predict that daughter d carries no mutant chromosome. It would therefore be possible to offer prenatal diagnosis to these parents using amniotic cells or chorionic villus samples to determine whether a future fetus was normal, a carrier for, or affected with the disease.

Figure 4 illustrates both the utility and the limitations of this type of analysis. This is a family in which the birth of a daughter to individuals 2 and 3 indicated that each parent carried the cystic fibrosis (CF) mutation. An extensive analysis for RFLP markers flanking the CF locus was carried out. A single marker (KM19) enabled tracking of the mutant paternal chromosome. However, no marker was informative for the mother (individual 3). Her brother (individual 4) could be clearly identified as a carrier, but it was not possible to distinguish his normal and mutant chromosome 7. A further problem in this family from the RFLP analysis was the inability to determine which grandparent of the affected child had contributed the mutant gene to her mother. This has relevance to genetic counseling of relatives from either the grandmaternal or grandpaternal side of the family.

Disease X need not be precisely within the fragment of DNA containing the polymorphism. Since human chromosomes are large (avg. ~100–150 × 10^8

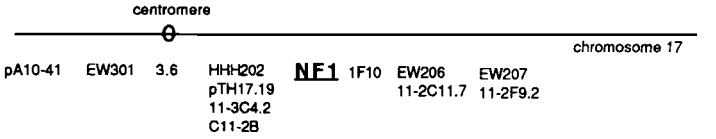
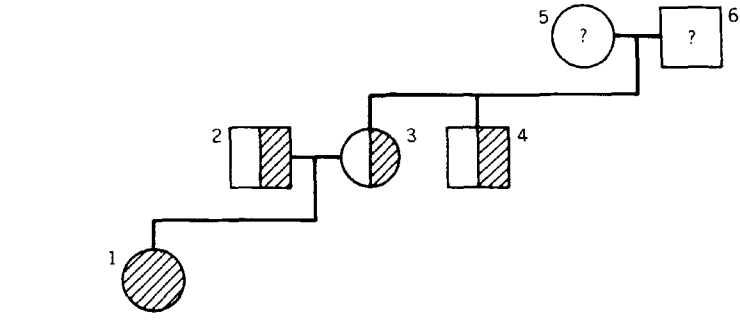


Fig. 3. Linkage analysis of selected RFLP markers associated with neurofibromatosis-1 (NF1) on chromosome 17.



	J3.11/MspI	1,1	1,1	1,1	1,1	1,2	1,1
	J3.11/TaqI	1,1	1,1	1,1	1,1	1,1	1,1
CF →	KM19/PstI	2,2	2,1	2,2	2,2	2,1	2,1
	XV2c/TaqI	1,1	1,1	1,1	1,1	1,2	1,2
	7C22/EcoRI	1,1	1,1	1,1	1,1	,	,
	MetH/MspI	2,1	2,2	1,1	1,1	,	,
	MetH/TaqI	2,1	2,2	1,1	1,1	,	,
	MetD/TaqI	1,2	1,1	2,2	2,2	,	,
	MetD/BanI	2,2	2,2	2,2	,	2,1	2,1
	5'Met/TaqI	2,1	2,2	1,1	1,1	,	,
	B79a/MspI	2,2	2,2	2,2	2,2	2,2	2,2

Fig. 4. RFLP analysis of cystic fibrosis-affected family.

nucleotides), and crossovers between two relatively close points are rare, RFLPs closely linked to known disease loci are excellent probes for genetic counseling and prenatal diagnosis. The accuracy of linkage predictions can be made with a probability that is related to the recombination frequency of an RFLP marker with the disease locus. If we had a second linked RFLP marker on the opposite side of X, the accuracy of risk prediction would become very high because it would require a double crossover event between both RFLP markers to redistribute a mutation to the opposite chromosome containing the apparently normal pattern. This is highly unlikely.

RFLP linkage analysis is the mainstay of genetic counseling and prenatal diagnosis for many inborn errors where the gene responsible for the disease has not yet been identified, such as Huntington's disease or myotonic dystrophy. RFLP markers, within and outside the gene, are still useful in diseases whose genes are identified when specific mutations (see below) cannot be identified for definitive testing; this includes many patients with Duchenne muscular dystrophy, hemophilia A and B, and neurofibromatosis. The determination of risk probabilities requires background in calculation techniques, and is best performed in most circumstances by sophisticated computer programs.

2.2.2. RESTRICTION ENZYMES TO DETECT DISEASE-CAUSING MUTATIONS

Rarely, the nucleotide sequence change responsible for a disease mutation alters the recognition sequence for a restriction enzyme, enabling the disease gene to be detected simply by the creation or abolition of a restriction endonuclease site. The classic example of direct detection is sickle cell anemia (Fig. 5); an A to T

mutation in the 6th codon of the beta globin gene abolishes an *Mst* II site, so that the mutant beta-S gene is marked by a larger restriction fragment. For other diseases in which multiple mutations may cause a mutant phenotype, such as Tay-Sach's disease (11) and the less common cystic fibrosis mutations (15, 16), a number lend themselves to direct detection, usually via PCR amplification and rapid gel analysis (Fig. 6).

In Duchenne muscular dystrophy the most common "mutations" are deletions of portions of the extremely large (>2 megabases) dystrophin gene (17–19). These deletions frequently contain RFLP sites leading to a loss of the RFLP band associated with the mutant X chromosome contributed by a carrier female to her son or daughter (Fig. 7).

Technical Notes. Different-sized alleles that constitute an RFLP locus may be detected in one of two ways.

1. The most common is by Southern (9) hybridization to a cloned DNA probe which recognizes sequences common to variably sized restriction fragments generated by the restriction enzyme. In this process, the DNA analyzed is separated by size on agarose gels and denatured, and the single-stranded molecules are transferred to a solid membrane. Hybridization of the membrane to labeled single-stranded molecular probes reveals fragments with homology to the probe which can be detected by autoradiography, colorimetrically, or with chemiluminescence. This procedure yields an answer in 4–7 days.

2. An increasingly popular method for DNA regions where the nucleotide sequence flanking the RFLP site is known is detection after polymerase chain reaction (PCR) amplification (10). In this procedure, oligonucleotide primers complementary to 5′–3′ sequences on both sides of the RFLP undergo a series of denaturation, annealing, and extension steps using a thermostable DNA polymerase and dNTPs, thus producing an exponential amplification of the DNA region (including the RFLP sequences) flanked by the primers. Digestion of the amplified fragment and analysis by agarose or polyacrylamide gel electrophoresis readily demonstrate the presence or absence of restriction endonuclease sites. The utility of multiple combinations of PCR primers to amplify up to 9 DNA regions simultaneously (multiplex PCR) has been demonstrated for assessing deletions in Duchenne muscular dystrophy (20, 21). While multiplex

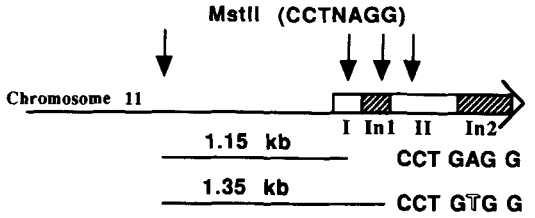


Fig. 5. Direct detection of sickle cell anemia using restriction endonuclease *Mst* II.

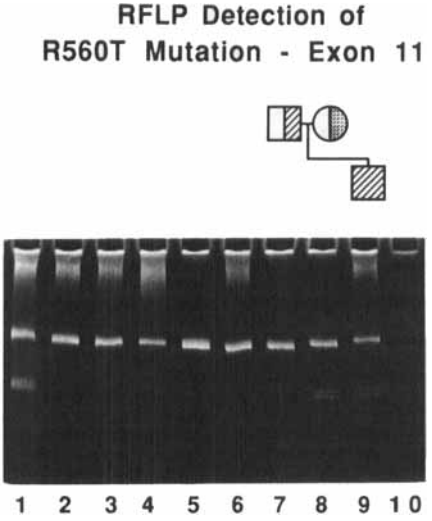


Fig. 6. Direct detection of cystic fibrosis mutation R560T using polymerase chain reaction and restriction enzyme *Mae*II. Nine patient samples and a minus DNA control (lane 10) were subjected to 30 cycles of polymerase chain reaction amplification for exon 11 of the cystic fibrosis transmembrane conductance regulator gene, digested with restriction enzyme *Mae*II, and electrophoresed on a polyacrylamide gel; the gel was stained with ethidium bromide. The 425 nucleotide fragment produced is the upper band in all patient samples. Lane 1 is from a patient known to carry the R560T mutation in which an arginine is changed to a threonine at amino acid residue 560. The nucleotide change responsible for this creates a *Mae*II restriction recognition sequence. Lanes 7–9 show a family in which the cystic fibrosis-affected son (9) has inherited a mutant R560T allele from his mother (8). The mutant allele contributed by the father (7) was another uncommon cystic fibrosis mutation.

analysis for restriction sites is theoretically possible, in practice reaction conditions for different enzymes, partial digestion products, and complexities of interpretation associated with multiple bands lead most laboratories do RFLPs by PCR one at a time.

2.2.3. HAPLOTYPES AND LINKAGE DISEQUILIBRIUM

In the analysis of family pedigrees it is usually possible by inspection of RFLP markers to "phase" or assign each to a specific chromosome. The array of RFLPs constitutes a *haplotype* unique to that chromosome. For many disorders, even those for which multiple mutations have been defined, within a given population or ethnic group a few specific mutations usually predominate (14). Generally each is associated with a distinctive haplotype of 2 or more RFLP markers. For example, functionally identical intervening sequence 1 splice site mutations in individuals of Mediterranean and Indian background carry completely different RFLP haplotypes (13). Similarly, two RFLP markers proved use-

ful in risk assessment of potential carriers prior to discovery of the cystic fibrosis transmembrane conductance receptor (CFTR) gene in 1989. Approximately 85% of CF mutations were associated with the XV2C/KM19 B haplotype [2.1 kb XV2C/*Taq*I,6.6 kb KM19/*Pst*I alleles]. Individuals with no family history of cystic fibrosis who started with the 1 in 25 population carrier risk could find their risk as

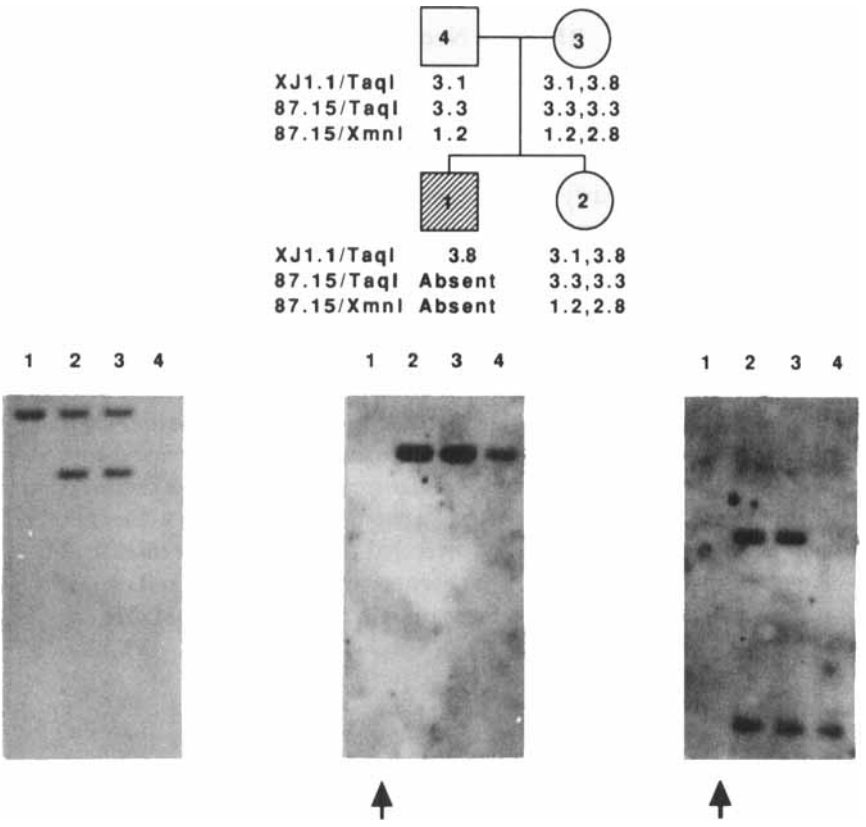


Fig. 7. RFLP analysis of Duchenne muscular dystrophy family. Preliminary analysis with 3 RFLP markers at the 5′ end of the dystrophin gene showed inheritance in the muscular dystrophy-affected son (1) of the maternal chromosome bearing the 3.8-kb XJ1.1/*Taq*I allele (*left*). DNA from the affected son showed no hybridization signal with the 87.15 probe using 2 different restriction enzyme markers (*Taq*I (*middle*) and *Xmn*I (*right*)), indicating deletion of sequences including the RFLP marker from this portion of the dystrophin gene. Subsequent studies with dystrophin gene cDNA probes confirmed these findings. A comparison of XJ1.1 marker patterns indicates that his sister has inherited the maternal X chromosome at risk and could be a carrier for Duchenne muscular dystrophy. However, she clearly has received a hybridizing 2.8-kb 87.15/*Xmn*I allele from her mother and shows no suggestion of decreased dosage of the noninformative 3.3 87.15/*Taq*I marker. Thus, she bears a low carrier risk. The hybridization signal for the 3.1-kb XJ1.1/*Taq*I allele in the father (4) is weak because less DNA was loaded.

low as 1 in 384 (CC haplotype) or as high as 1 in 5 (BB haployte) after haplotype analysis (12).

Not surprisingly, the impact of haplotype contribution to risk assessment declines significantly with the ability to identify specific disease mutations. Note that the ominous 1 in 5 risk of a BB haplotype in individuals reporting no family history of cystic fibrosis has now fallen to 1 in 60 with the ability to assay directly for mutations comprising approximately 85% of all CF carriers (Table 3).

2.3. RFLPs in Neoplasia and Cancer

2.3.1. LOSS OF RFLP HETEROZYGOSITY AND TUMOR SUPPRESSOR GENES

The comparison of RFLP patterns between normal and tumor tissue has provided compelling evidence in support of a multihit theory for carcinogenesis (22, 23), an explanation for early and often multisite occurrence of hereditary cancer, and insight into a broad new group of regulatory genes whose dysfunction can lead to neoplasia. The crucial observation occurred in patients heterozygous for RFLPs whose tumor tissue showed a single RFLP rather than the heterozygous pattern of normal tissues. This phenomenon, termed loss of heterozygosity (LOH), has been demonstrated for an increasing number of tumors (Fig. 8, Table 4). In some cases, losses were not unexpected because of cytogenetic deletions in such tumors as retinoblastoma (del 13, q1.4) and Wilm's tumor (del 11, p1.3). In many others, such as colon carcinoma, losses were not restricted to a single chromosome, but were quite extensive. Despite broad LOH, a predilection for losses on chromosomes 17p, 18q, and 5q was noted along with a clinical correlation suggesting that greater LOH portended more aggressive disease (24).

The operative concept for genetic events correlating LOH and cancer is that the physical deletion of genetic information in regions showing LOH causes the

TABLE 3

RFLP Haplotype Contribution to CF Carrier Risk

	Carrier Risk	
XV2C/KM19 Haplotype	No mutation analysis	85% mutations detectable
AA	1 in 166	1 in 249
BB	1 in 5.2	1 in 62
CC	1 in 214	1 in 220
DD	1 in 71	1 in 176
AB	1 in 9.2	1 in 99
AC	1 in 187	1 in 234
AD	1 in 100	1 in 206
BC	1 in 10.2	1 in 97
BD	1 in 8.9	1 in 92
CD	1 in 106	1 in 196

Source: Beaudet et al (12) and A.L. Beaudet, personal communication.

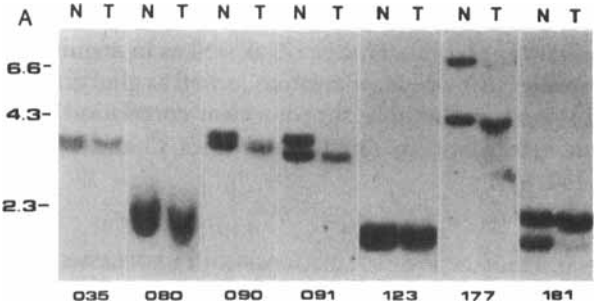


Fig. 8. Loss of RFLP heterozygosity in tumor tissue. Hybridization of probe p68RS2.0 for a variable number of tandem repeats (VNTR) marker on chromosome 13 to normal (N) and breast tumor (T) tissue is demonstrated for 7 patients. Note the presence of 2 bands in normal tissue from each patient, but markedly decreased or absent hybridization of either the upper or lower allele in a number of patients, most clearly shown for patients 035 (lower), 090 (upper), 091 (upper), 177 (upper), and 181 (lower). Numbers to the left indicate the size (in kb) of marker fragments. (From Devilee et al. (57), reproduced with permission from Academic Press.)

TABLE 4

Some Tumors Demonstrating Loss of Heterozygosity (LOH)

Acoustic neurofibroma
Acute myeloid leukemia (AML)
Astrocytoma, high grade
Bladder carcinoma
Breast carcinoma
Colon carcinoma
Lung carcinoma, small cell
Lung carcinoma, other
Melanoma (standard and uveal)
Meningioma
Multiple endocrine neoplasia, types 1 and 2
Neuroblastoma
Neurofibrosarcoma
Osteosarcoma
Retinoblastoma
Rhabdomyosarcoma
Soft tissue sarcoma
Wilm's tumor, sporadic and familial

loss of tumor suppressor genes or anti-oncogenes (25), which help maintain orderly cell growth. Since most tumor suppressor genes are autosomal, the loss of function of 1 gene may be compensated in many cases by the remaining gene. However, inheritance of 1 mutant gene leaves no reserve should the other become mutated; this is felt to be the basis for hereditary cancers. Actual loss of gene function need not necessarily be large-scale deletion of genetic material as

noted in LOH, but could take the form of point mutations, as occur in the p53 gene in hereditary breast cancer (26, 27) as well as in acquired hepatomas (28, 29). Further studies using retinoblastomas as well as glial tumors of varying histologic and biologic grades have shown a clear correlation between increasing loss of genetic heterozygosity on chromosomes 13 and 10 and histologic aggressiveness (30, 31).

2.3.2. CLONALITY ANALYSIS OF HEMATOLYMPHOID NEOPLASMS

Production of functional immunoglobulin or T-lymphocyte antigen receptor genes is associated with commitment to lymphoid differentiation. It requires a unique series of molecular events in which small portions of the chromosome are lost by rearrangement of germline immunoglobulin and T-cell antigen receptor genes linking variable (V) with joining (J) as well as diversity (D) segments. The constant region (C) is not involved. A mature mRNA for translation is produced by splicing of the intron from between the J and C domains. An example of this for the immunoglobulin light chain genes is depicted in Fig. 9. Associated with this process, DNA between rearranged V and D or J segments of these genes is lost, with changes in the length of restriction endonuclease fragments. In Fig. 9, the *Eco*R1 restriction fragment in the rearranged allele has become larger than the germline allele. When there is proliferation of a single clone of lymphoid cells, as in lymphoma or leukemia, enough new fragments of one species are present to give a new hybridizing band on Southern blots (Fig. 9*a*, neoplastic). Because first attempts at lymphoid commitment are sometimes unsuccessful, rearrangement may occur on both chromosomes, so that two new hybridizing fragments can be seen (Fig. 9*b*).

The ability to detect clonal populations has provided insights into some processes and sparked controversy in others. Two examples of insights are the demonstration that most childhood acute lymphoblastic leukemias are of pre-B lymphoid origin (44) and the demonstration of clonal rearrangements of T cell antigen beta-chain receptor genes in cases of "dermatopathic" lymphadenopathy in patients with the cutaneous T-cell proliferation, mycosis fungoides. It has long been known that patients with palpable lymphadenopathy have a more aggressive course than those without, and yet clearcut lymphomatous involvement frequently cannot be defined histologically. Conversely, the description of faint clonal immunoglobulin rearrangements in patients with Hodgkin's disease has led to varying interpretations of rearrangements as an indication that the underlying neoplastic Reed–Sternberg cells are of lymphoid origin to a localized oligoclonal but benign reaction by normal lymphoid cells (47, 48).

In polyclonal reactive processes, there may be some diminution in hybridization signal strength of the germline alleles due to rearrangement at this locus. Since a single clone of lymphoid cells is not present, there are not enough rearranged alleles of any one type to give a new band. Hence, clonality is synonymous with a clonal proliferation. Interestingly, clonality does not always connote malignancy; a good example is the indolent T-gamma or T8 lymphocytosis syndrome. Examples have also been described of small clonal populations felt to

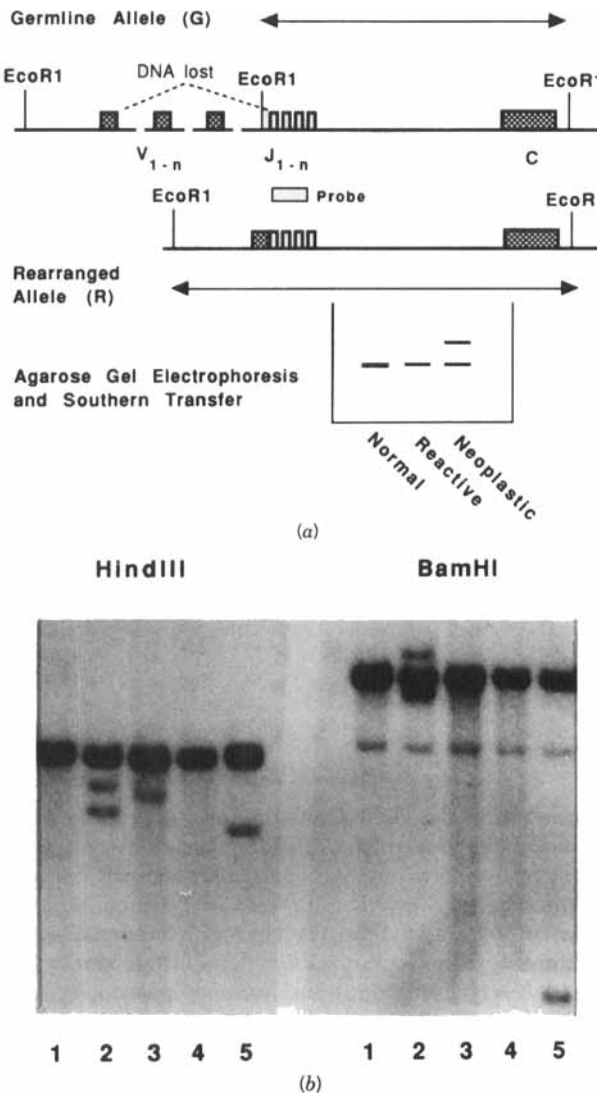


Fig. 9. (*a*) Immunoglobulin light chain gene rearrangement. See text for details. (*b*) Immunoglobulin heavy chain gene rearrangement studies in 5 patients. DNA from 5 patient samples was extracted, digested with restriction enzymes *Hin*dIII and *Bam*HI, size-fractionated on agarose gels, Southern transferred and hybridized to a joining region (J) probe for the immunoglobulin heavy chain gene on chromosome 14. Each sample demonstrates a single strongly hybridizing germline allele of 9.5 (*Hin*dIII) and 17 (*Bam*HI) kb in size. Samples 2, 3, and 5 show rearranged alleles. Sample 5 demonstrates a single rearrangement with each enzyme, while patient 2 demonstrates 2 rearranged alleles. The strongly hybridizing germline band in samples 2, 3, and 5 suggests that the clonal lymphoma population comprises a modest fraction of the total cells sampled. Sample 3 also clearly exhibits a new allele on the *Hin*dIII digest, but no new alleles with *Bam*HI; presumably the new allele comigrates with the germline fragment on the *Bam*HI digest.

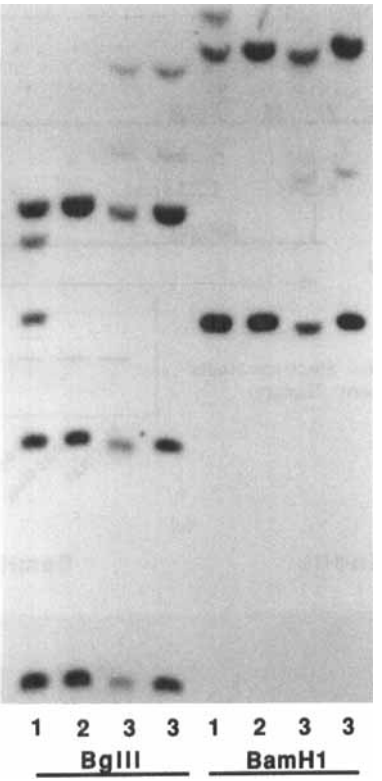


Fig. 10. Demonstration of the Philadelphia chromosome using restriction enzyme analysis. DNA from 3 patients was digested with restriction enzymes *Bgl*II and *Bam*HI, size-fractionated on agarose gels, and hybridized after Southern transfer to a probe for the breakpoint cluster region (bcr) on chromosome 22. Patient 2, a known normal, shows a characteristic pattern of 3 and 2 hybridizing bands with these enzymes. Patients 1 and 3, who have chronic myelogenous leukemia, both demonstrate new hybridizing bands (2 with *Bgl*II and 1 with *Bam*HI), reflecting translocation of a portion of chromosome 9 into this region, and with it a change in the size of hybridizing restriction fragments, much like the process portrayed for immunoglobulin genes (see Fig. 9). The normal pattern of alleles also remains because there is retention of 1 normal nontranslocated chromosome 22.

represent nonneoplastic restricted antibody responses to reactive stimuli. Molecular studies that detect clonally rearranged immunoglobulin or T-cell antigen receptor genes are useful diagnostically in some of the following circumstances:

- To distinguish reactive "atypical" lymphoid hyperplasia from lymphoma
- To distinguish Hodgkin's from non-Hodgkin's lymphomas
- To distinguish poorly differentiated carcinoma from large cell lymphoma
- To demonstrate the presence of small populations of lymphoma cells (as few as 2–5%) in specimens

Rearrangements of the T-cell receptor (TCR) beta-chain gene are particularly helpful in assessing clonality in T cell lymphomas, where, unlike the situation with light chain restriction in B cell lymphomas, immunologic assessment of clonality can be very difficult. Rare leukemias and lymphomas may arise from gamma–delta T lymphocytes which may be detectable only by molecular probes to these genes, while the T cell receptor beta-chain gene retains a germline configuration (32). T cell receptor gamma- and delta-chain genes also rearrange in a variety of B-cell and myeloid leukemias (32, 45, 46), and can be used as clonal markers for diagnosis and detection of minimal residual disease (45).

2.3.3. CONSISTENT CHROMOSOMAL TRANSLOCATIONS

Restriction enzyme analysis also offers a precise way to look for specific quantitative and qualitative chromosomal abnormalities which are restricted to defined chromosomal areas. Consistent molecularly detectable breakpoints within small chromosomal regions such as the 9;22 Philadelphia chromosome (51, 52) (Fig. 10), the 15;17 translocation of acute promyelocytic leukemia (49, 50), and the 8;14 (c-myc) or 14;18 (bcl-2) translocations of non-Hodgkin's lymphomas (53–56) are easily detectable on Southern blots. Breakage of the chromosome within a restriction endonuclease fragment detected by a specific molecular probe invariably leads to a single new allele of larger or smaller size. While lacking the global overview of conventional cytogenetics, restriction enzyme analysis for specific breakpoints offers a number of unique advantages for the detection of specific breakpoints in specimens where cytogenetics fails, is confusing, or cannot be performed (such as frozen samples).

2.3.4. CLONALITY STUDIES OF NONHEMATOLYMPHOID TUMORS

While rearranging genes of hematolymphoid tumors are tailor-made for clonality studies, with the exception of rare cytogenetic or molecular genetic markers, no similar approach exists for the majority of neoplasms. One attempt to analyze these tumors has focused on the study of tumors in women using the Lyon hypothesis. The hypothesis predicts that in early embryogenesis, one of the two X chromosomes in each female cell will undergo random inactivation, and this feature becomes a heritable trait passed to all progeny of that cell (33). Thus, lesions whose origin is questionable between polyclonal hyperplasia or monoclonal neoplasia lend themselves to analysis via the X chromosome. One limitation, of course, is that this restricts the study population to women, and moreover to women in whom the two X chromosomes can be easily distinguished functionally. Glucose-6-phosphate dehydrogenase, an X chromosome gene, has contributed much valuable information as a prototype marker for such studies (34). However, its distribution is restricted to a small percentage of black females.

Another approach has been to utilize X chromosome RFLP markers that physically undergo changes in methylation associated with inactivation of the X chromosome (35–37). The RFLP marker enables discrimination of each chromosome, while digestion with restriction enzymes that do or do not digest

methylated DNA allow the investigator to tell if both alleles from a lesion are digested proportionally, suggesting a polyclonal derivation of the specimen, or disproportionately, suggesting a monoclonal origin. In this fashion, a monoclonal origin has been convincingly shown for adenomatous and villus polyps in the colon (38), thyroid (39, 40) and parathyroid (41) adenomas, and even remission bone marrows in certain cases of acute myelogenous leukemia (42) (Fig. 11).

2.4. Infectious Diseases

A particularly useful laboratory application of restriction enzymes is in the epidemiologic investigation of bacterial and plasmid isolates to see if patient isolates reflect personnel- or equipment-based sources of infection (43). A fruitful approach to this issue has been to use restriction enzymes and probes to study the "fingerprint" pattern of ribosomal rRNA genes, which because of their large number will demonstrate characteristic identifying patterns even within strains.

3. OVERVIEW

Restriction enzymes have become a key reagent for many biomedical research applications and their place in the future of biomedical research and clinical

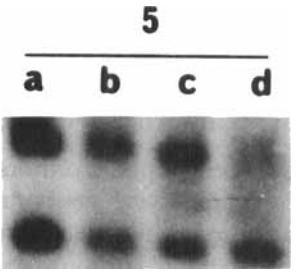


Fig. 11. Clonal analysis of thyroid follicular nodules using X chromosome RFLPs and methylation-sensitive restriction endonucleases. DNA from a woman's peripheral blood (lanes a and b) and adenomatous thyroid nodule (lanes c and d) was analyzed using restriction digestion, Southern transfer, and hybridization to a probe for the phosphoglycerate kinase (PGK) gene, which is located on the X chromosome. Lanes a and c show that both the peripheral blood and tumor specimen are heterozygous for PGK RFLP alleles of 1.8 and 1.4 kb. Lanes b and d represent similar treatment, with an additional predigestion using restriction enzyme *Hpa*II, which cuts DNA at certain sequences that have been methylated. Methylation is associated with inactivation of the X chromosome in cells of females. Inactivation is a random process that occurs early in embryogenesis and then becomes an immutable property of each cell. Thus, the roughly equal loss of hybridization intensity for each RFLP allele in the peripheral blood sample (lane b) is consistent with an expected polyclonal population containing a mixture of inactivated X chromosomes. By contrast, in the tumor sample, only the 1.8-kb allele shows digestion, indicating that cells in the tumor population all contain the same inactive X chromosome, and presumably are a clonal population derived from a single progenitor.

applications seems assured, at least for the next several decades. Their specificity enables the isolation and cloning of virtually any discrete nucleic acid region, and the use of oligonucleotides with adapter restriction enzyme recognition sequences allows great flexibility in the placement of utilitarian restriction sites.

If anything, the importance of RFLP markers is likely to increase with the wealth of nucleotide sequence data that will be provided by automated sequencing technologies and human genome initiatives throughout the world. From this information, along with yeast artificial chromosome (YAC) and cosmid contig libraries, there will almost certainly be an exponential addition of genetic diseases whose chromosomal location is determined. RFLP markers will remain a primary diagnostic test for genetic counseling of patients with such disorders. Most such tests will be done with polymerase chain reaction approaches using restriction enzymes to assess products and nonradioactive detection systems. An exciting development in this regard is the emergence of fluorescently labeled oligonucleotides which become part of the PCR reaction product and thus restriction fragments generated for analysis. These promise to offer capabilities for automated analysis, perhaps in batch mode, rather than the more laborious manual methods currently employed.

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Advances in Enzymatically Coupled Field Effect Transistors

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1. INTRODUCTION

The first biosensor based on semiconductor technology was reported by Caras and Janata in 1980 (1). They developed a microbiosensor sensitive to penicillin based on a hydrogen ion-sensitive field effect transistor (FET) transducer in conjunction with a penicillinase-immobilized membrane. This type of biosensor offers discriminating advantages over the conventional counterpart with an electrode transducer (see Chapter 3):

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1. *Micro size:* potential for in vivo application
2. *Solid state:* no internal liquid
3. *Multifunctionality:* simultaneous determination of more than two chemicals with a single biosensor
4. *High mass productivity:* possibility for making an inexpensive and eventually disposable biosensor
5. *Integration:* incorporation of signal amplification and processing functions in the semiconductor chip

The hydrogen ion-sensitive FET is the most frequently used transducer for the semiconductor biosensor. It was initially developed for in vivo hydrogen ion concentration measurements (2–4). Other kinds of FET are made that are sensitive to other ionic species, for example, potassium, sodium, and calcium (5). In an enzymatically coupled FET biosensor, an enzyme immobilized on a membrane is attached to the hydrogen ion-sensitive gate of a FET. The enzyme brings about a change in hydrogen ion concentration when its substrate is decomposed in the membrane. The hydrogen ion concentration change is then transduced into an electrical signal by the FET. Several enzymatically coupled FETs have been reported, for example, glucose- (6–23) and urea-sensitive FET biosensors (8, 9, 13, 15–18, 20, 24, 25) and FET biosensors sensitive to a neutral lipid (26), acetylcholine (25), L-glutamate (27) and so on.

Semiconductor fabrication techniques have also been successfully applied to the construction of conventional transducers sensitive to hydrogen peroxide, oxygen, and carbon dioxide. A hydrogen peroxide-sensitive silicon chip was made by using metal deposition techniques (28, 29). The combination of the hydrogen peroxide-sensitive transducer and enzyme-immobilized membranes gave a miniaturized and multifunctional biosensor. Similarly, an oxygen- and a carbon dioxide-sensitive device was made and applied to the construction of biosensors (25, 30, 31).

The enzymatically coupled FET is reviewed in this chapter (32). First, a brief review is given. Determining how to deposit an enzyme-immobilized membrane on the surface of a FET was one of the most difficult problems to solve before an enzymatically coupled FET could be developed. Therefore, some enzyme-immobilized membrane deposition methods and the photolithographic enzyme-immobilized membrane patterning method developed by the authors are described in detail. Concomitantly, the performances of some FET biosensors with an enzyme-immobilized membrane made by this method are described. Finally, recent applications of an enzymatically coupled FET are surveyed.

2. REVIEW OF ENZYMATICALLY COUPLED FET

2.1. Structure of Ion-Sensitive FET

The basic structure of an ion-sensitive FET is illustrated in Fig. 1*a*. The silicon chip has three principle components: a drain (D), a source (S), and an ion-

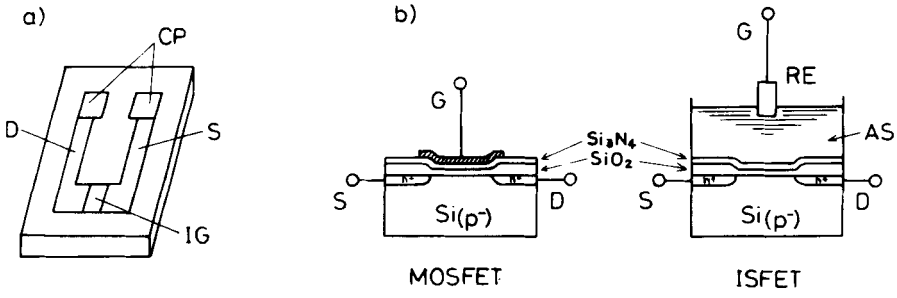

Fig. 1. (*a*) Structure of an ion-sensitive FET transducer with single FET element. (*b*) Structure of an ion-sensitive FET with metal oxide semiconductor FET. D, drain; S, source; IG, ion-sensitive gate; CP, contact pad; G, gate; RE, reference electrode; AS, analyte solution; MOSFET, metal oxide semiconductor FET; ISFET, ion-sensitive FET.

sensitive gate (IG). Its cross-sectional view is schematically compared with a metal oxide semiconductor field effect transistor in Fig. 1*b*. The metal oxide FET is an electronic part that utilizes the gate potential to control the electric current between the source and drain. An ion-sensitive FET has no gate electrode; instead, it has an external reference electrode.

Silicon nitride is typically used as the material for a hydrogen ion-sensitive gate, but tantalum oxide and aluminum oxide can also be used (4). FETs sensitive to other ionic species use other ion-sensing membranes (5). The reader is directed to reviews (33–35) of the fabrication, function, and operation of ion-sensitive FETs.

A brief description follows as to how a hydrogen ion- sensitive FET works as a pH sensor. The outer surface of the silicon nitride layer is covered by a thin layer of silicon oxide, which has silanol groups in an aqueous solution. The proton dissociation of the silanol groups is a function of the hydrogen ion concentration of a solution. Because the dissociation of one proton leaves a negative charge on the outer surface of the ion-sensitive gate, an interfacial potential is created between the ion-sensitive gate and a solution. The interfacial potential controls the current flow from the source to the drain through "field effect." Thus, a hydrogen ion-sensitive FET utilizes the interfacial potential to detect the hydrogen ion concentration of a solution. A hydrogen ion-sensitive FET shows linear response over fairly wide pH range, with sensitivity of 50–60 mV/pH at ambient temperatures.

A transistor-based chemical sensor used in aqueous environment must be electrically insulated. An ordinary FET sensor produced with a silicon wafer as starting material has four bare lateral edges, which are made when scribing a processed silicon wafer into FET chips. It has bonding pads on its surface for wires that are connected for the electrical operation of a FET (see Fig. 1*a*). It is necessary to insulate the bare lateral sides and bonding pad region, and this is usually done by polymer encapsulation. Because a FET chip is very small and the areas to be insulated are closely located to its ion-sensitive gates, which must

remain exposed, it is necessary to insulate by a tedious manual process. Overcoming the polymer encapsulation problem is necessary before the mass production of a FET chemical sensor can be economical (36).

Two major improvements in the fabrication of an ion-sensitive FET that avoid most of the tedious polymer encapsulation process have been reported. Matsuo and his coworkers (4, 37) fabricated a probe-type FET with a three-dimensional silicon nitride passivation layer around most of its surface, as shown in Fig. 2. The probe-type FET has one disadvantage: Its fabrication requires a three-dimensional process that is uncommon for semiconductor construction facilities. An alternative approach utilizes a silicon-on-sapphire (SOS) wafer for FET fabrication (38, 39). The structure of a SOS-FET is depicted in Fig. 3. It has an island-like silicon layer on a sapphire substrate, in which an ion-sensitive FET is fabricated. The bare lateral sides do not need encapsulation because of the high insulation property of sapphire.

2.2. Structure of Enzymatically Coupled FET

An enzymatically coupled FET requires two independent hydrogen ion-sensitive FET elements: One carries an enzyme-immobilized membrane on its surface, called an enzyme FET, and the other, with no enzymatic activity, is a reference FET. Figure 4 is a schematic representation of an enzymatically coupled FET (shown in the area surrounded by the broken circle). The enzyme immobilized on the surface of the enzyme FET (EIM in Fig. 4) causes a change in

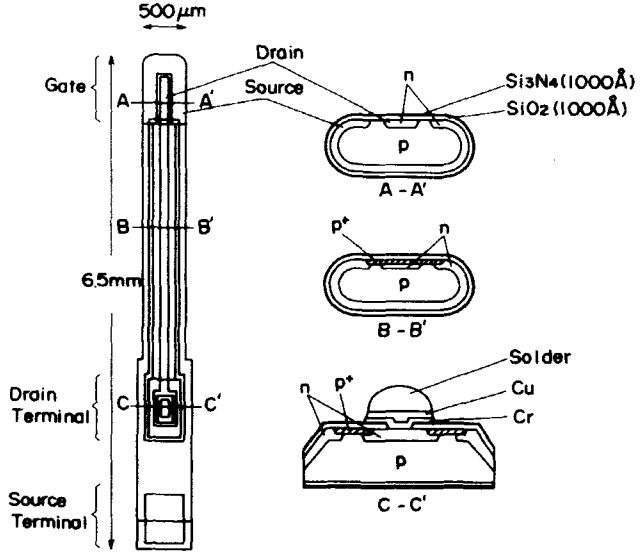

Fig. 2. Structure of a probe-type FET transducer with a silicon nitride layer surrounding the majority of the surface. (Reproduced from Matsuo and Esashi (37), with permission.)

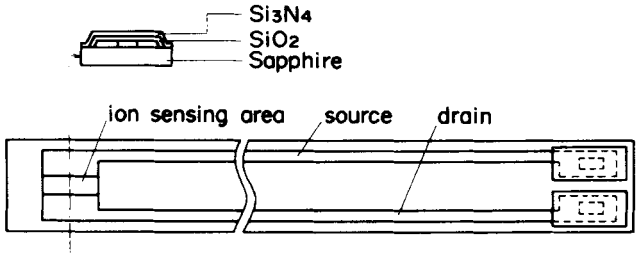

Fig. 3. Top and cross-sectional views of a FET transducer fabricated with a silicon film on a sapphire wafer (SOS–FET). (Reproduced from Akiyama et al. (38), with permission.)

the hydrogen ion concentration when its substrate is decomposed, and thus changes the output voltage of the enzyme FET. The reference FET, on the other hand, which has a membrane with no enzymatic activity (MNE), gives no change in its output voltage. Therefore, the enzyme substrate can be detected by measuring the differential output voltage between the two FETs.

The reference electrode (RE) shown in Fig. 4 provides a fixed constant potential to the solution. A liquid containing reference electrode with a stable potential, such as a silver/silver chloride electrode, is used for this purpose. As will be discussed in the next section, an inert metal such as platinum or gold can also be used. The electrical circuit for the operation of the two FET elements and the differential output measurement is shown in Fig. 4.

The glucose-sensitive, enzymatically coupled FET has been most extensively studied. The immobilized glucose oxidase utilizes molecular oxygen to oxidize glucose to produce gluconic acid via glucono-δ-lactone. The gluconic acid increases the hydrogen ion concentration in the enzyme-immobilized membrane. Consequently, glucose in a sample solution can be determined by observing the differential output between the enzyme FET and reference FET.

If one wants to make an enzymatically coupled FET that is simultaneously sensitive to n chemicals, called a multifunctional FET biosensor, $n + 1$ FET elements are needed for its fabrication: one reference FET and n enzyme FETs with a different enzyme-immobilized membrane on each gate surface of the n FETs. Each analyte can be detected independently by measuring the differential output voltage between the reference FET and the corresponding enzyme FET.

2.3. History of Enzymatically Coupled FET

The first enzymatically coupled FET is sensitive to penicillin (1). It is composed of two separate FET chips and a silver/silver chloride reference electrode (see Fig. 4). Penicillinase, which produces protons during catalysis, is immobilized on one of the FET chips by utilizing the glutaraldehyde protein cross-linking reaction. The membrane of the reference FET chip contains immobilized bovine serum albumin.

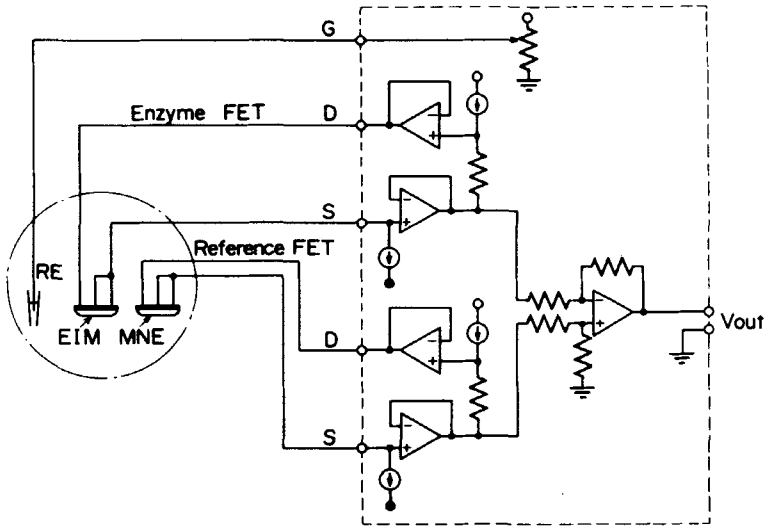

Fig. 4. Schematic representation of an enzymatically coupled FET (broken circle) with electrical circuit for differential output measurement (dashed line): EIM, enzyme-immobilized membrane; MNE, membrane with no enzymatic activity; RE, reference electrode; G, gate; D, drain; S, source. (Reproduced from Hanazato et al. (10), with permission.)

The next two clinically important chemicals to be analyzed by an enzymatically coupled FET were glucose and urea. Glucose- and urea-sensitive FET biosensors were made, adopting almost the same configuration as the penicillin-sensitive FET (6, 8). The structure of these FET biosensors is shown in Fig. 5. The sole difference between the two biosensors and the penicillin biosensor is that a platinum metal electrode replaces the silver/silver chloride electrode. It was confirmed experimentally that an inert metal could be employed as a pseudo reference electrode for the enzymatically coupled FET, instead of the reference electrode, because the instability of the metal/liquid interface potential could be cancelled out by simply measuring the differential output between two FET elements (6). Using an inert metal as a pseudo reference electrode is more advantageous than an ordinary liquid-containing electrode. A metal electrode is structurally simple, requires no tedious preparation work, and is easily made by common mass-production methods.

Table 1 summarizes the characteristics of several additional FET enzyme sensors. Enzymatically coupled FETs sensitive to acetylcholine (25), triglyceride (26), adenosine triphosphate (ATP) (40), adenosine diphosphate (ADP) (41), glutamate (27), and threonine (42) have been made.

A multifunctional enzymatically coupled FET was first described by Hanazato et al. (8). They fabricated a bifunctional enzymatically coupled FET which

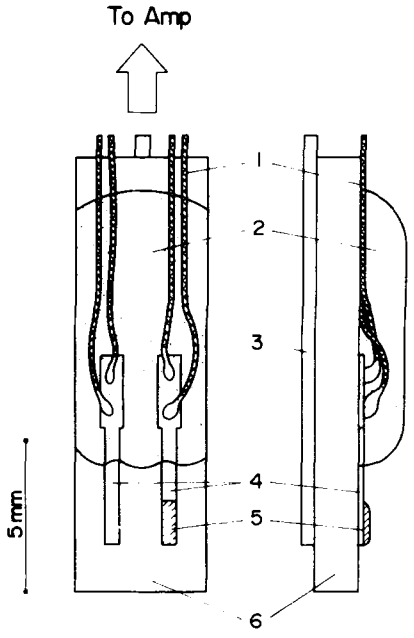

Fig. 5. Structure of FET sensor with two discrete FET chips: 1, connecting wire; 2, epoxy resin; 3, platinum wire; 4, FET chip; 5, enzyme-immobilized membrane; 6, epoxy laminate. (Reproduced from Nakako et al. (26), with permission.)

TABLE 1

Enzymatically Coupled FET

Analyte	Enzyme	References
Penicillin	Penicillinase	1
Glucose	Glucose oxidase	6-23
Urea	Urease	8, 9, 13, 15-18, 20, 24, 25
Triglyceride	Lipase	26
Acetylcholine	Acetylcholine esterase	25
ATP	ATPase	40
ADP	Pyruvate kinase	41
Glutamic acid	Glutamate decarboxylase	27
Threonine	Threonine deiminase	42

allowed independent or simultaneous determination of glucose and urea. It had three separate FET chips: two enzyme FETs containing a glucose oxidase- and a urease-immobilized membrane, and one reference FET. Platinum metal was used as a pseudo reference electrode (see Fig. 5). The differential outputs between the two enzyme FETs and the reference FET gave glucose and urea responses. The response curves of the bifunctional FET are depicted in Fig. 6. The upper traces show glucose responses and the lower traces show urea responses. Note that glucose has little effect on the urea response and vice versa.

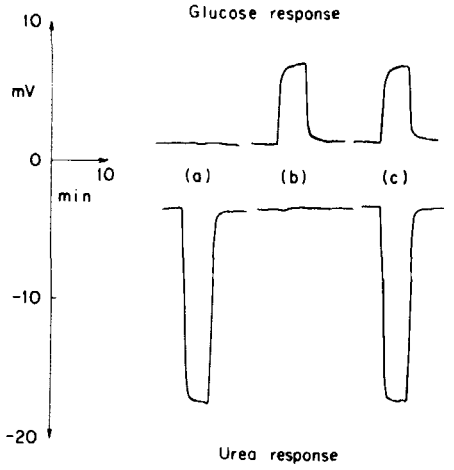

Fig. 6. Response curves of a bifunctional FET sensor sensitive to urea and glucose. Response curves for (*a*) 8.3 mM urea solution, (*b*) 3.1 mM glucose solution, and (*c*) solution containing 8.3 mM urea and 3.1 mM glucose. (Reproduced from Hanazato et al. (8), with permission.)

While the enzymatically coupled FETs described so far use separate FET chips, more advanced forms, whether monofunctional or multifunctional, use an integrated FET chip with more than two working FET elements. The bifunctional enzymatically coupled FET described above is not truly multifunctional because it does not use an integrated FET transducer. The advanced type of FET biosensor is called a monolithic enzymatically coupled FET (43). After the bifunctional enzymatically coupled FET, we have used an integrated FET chip shown in Fig. 7, which has three independently functioning FET elements. The dimension of the FET chip is $5 \times 6.5 \times 0.5$ mm. The ion-sensitive gate is 0.02 mm wide and 1 mm long.

A monolithic monofunctional enzymatically coupled FET sensitive to urea was first fabricated by Kuriyama and Kimura's group (44). Their integrated FET chip has two hydrogen ion-sensitive FET elements. One FET element has a urease-immobilized membrane, working as an enzyme FET, and the other has a ultraviolet (UV) light-inactivated, urease-immobilized membrane (see Section 3), working as a reference FET.

A monolithic bifunctional enzymatically coupled FET simultaneously sensitive to urea and glucose was made by Miyahara et al. (20), using an integrated FET chip with three FET elements. The center FET element with no enzyme activity is used as a reference FET; the right and left FET elements have a urease- and a glucose oxidase-immobilized membrane on their ion-sensitive gate surfaces. Neither urea or glucose affected the response of the FET to the alternate compound. Furthermore, Kuriyama and Kimura's group made a trifunctional FET chemical sensor, combining two enzyme biosensors and one ion sensor (15). Using this trifunctional FET made it possible to determine urea, glucose, and potassium concentrations simultaneously.

Computer simulation of the time course of the pH change created by the enzyme substrate reaction shows that the dependence of the response amplitude

on the buffering capacity of the analyte is one of the disadvantages of an enzymatically coupled FET (14, 19, 21, 22, 24). To overcome this disadvantage, Van Der Schoot and Bergveld (23) developed a new structure for the enzymatically coupled FET. They introduced a feedback mechanism to compensate for the pH change created by the enzymatic reaction by incorporating hydrogen or hydroxy ion generating electrodes on the surface of the FET. They show that the electrode current necessary for compensating pH change depends on analyte concentration and that the new FET biosensor functions as a chemical sensing device, its response being independent of buffering capacity.

3. PHOTOLITHOGRAPHIC PATTERNING OF ENZYME-IMMOBILIZED MEMBRANES FOR ENZYMATICALLY COUPLED FET

The advanced type of enzymatically coupled FET utilizes an integrated FET transducer chip with several FET elements closely spaced to each other (see Section 2.3 and Fig. 7). The monolithic enzymatically coupled FET requires that small and well-defined enzyme-immobilized membranes be patterned on the specific areas of such a FET chip. In addition, the method for depositing the enzyme on the membrane should be compatible with mass-production processes. Therefore, a more sophisticated procedure is needed to deposit enzyme on the membranes used in the monolithic enzymatically coupled FET.

Photolithographic methods, which play a key part in the fabrication of semiconductors, are potential candidates for the photo-patterning of small enzyme-immobilized membranes on a FET at its wafer stage. Ion-sensing FET devices with neutral carrier membranes sensitive to alkaline and alkaline earth metal

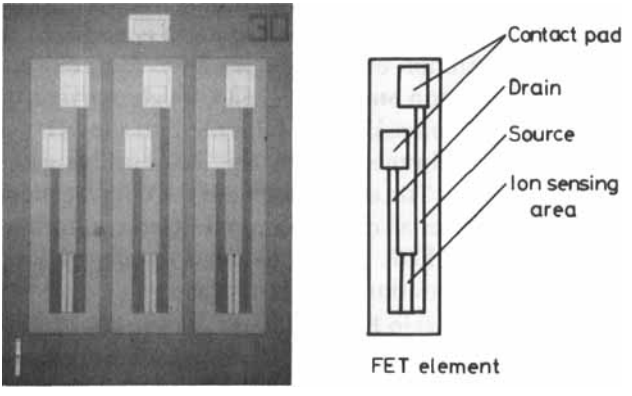

Fig. 7. *Left:* Photograph of a monolithic FET transducer with three independently functioning FET elements. *Right:* Structure of one FET element. (Reproduced by permission of CMC Press, Tokyo, Japan.)

ions have already been constructed with photolithographic membrane deposition methods (5, 45). Using a commercially available photoresist made it possible to construct small ion-sensitive membranes.

Several photolithographic methods have been proposed for depositing enzyme-immobilized membranes on a FET surface (7, 9, 10–13, 15–17, 20, 28, 43, 44). The original photolithographic method utilized the partial inactivation of an enzyme-immobilized membrane formed on the whole FET surface, by UV irradiation through a photomask. It was used for the urea-sensitive FET described in Section 2.3 (44). An enzyme-immobilizing solution containing glutaraldehyde as a chemical cross-linking agent is spin-coated on the whole surface of a FET. After the cross-linking reaction in the spin-coated membrane is complete, the enzyme-immobilized membrane is UV irradiated through a photomask; the photomask permits UV light to go through to the whole area of the enzyme-immobilized membrane except the area defining the ion-sensitive gate surface of the enzyme FET. The wavelength of the UV light is between 250 and 300 nm, so that the UV irradiated part of the enzyme immobilized membrane loses its enzymatic activity. Consequently, the enzyme FET has an active enzyme-immobilized membrane, while the reference FET was enzymatically inactive.

The selective UV-inactivation method is effective for the deposition of a single enzyme-immobilized membrane on a FET, but it is not possible to deposit several different enzyme-immobilized membranes on one FET chip. Other photolithographic methods, outlined in Fig. 8, do not have this disadvantage. The methods are applicable to the simultaneous deposition of different membranes on a FET chip. In other words, these methods are usable for the preparation of a multifunctional enzymatically coupled FET.

Brief descriptions of the three methods are given as follows:

1. *Direct Photolithographic Patterning* (see (1) in Fig. 8) (7, 9, 10, 13). This method uses light-sensitive, water-soluble compounds, which following UV irradiation form a three-dimensional insoluble polymer to entrap enzyme molecules. An enzyme-immobilizing solution is prepared by dissolving the enzyme in a solution of the light-sensitive compound, followed by spin-coating the solution on a FET. Then UV light is irradiated through a photomask. The photomask used for this method allows UV light to reach the spin-coated membrane only on the ion-sensitive gate area of the enzyme FET element. The development of the enzyme membrane in an aqueous solution leaves an enzyme-immobilized membrane only on the surface of the enzyme FET. The UV light has wavelength longer than 350 nm to minimize inactivation of the enzyme.
2. *Micropool Injection* (see (2) in Fig. 8) (15, 16, 20, 43). Micropools are photolithographically formed using a light sensitive film on the ion-sensitive gates of FET elements. The UV light-sensitive film is first spin-coated onto the FET and then irradiated through a photomask. Finally, the film resist

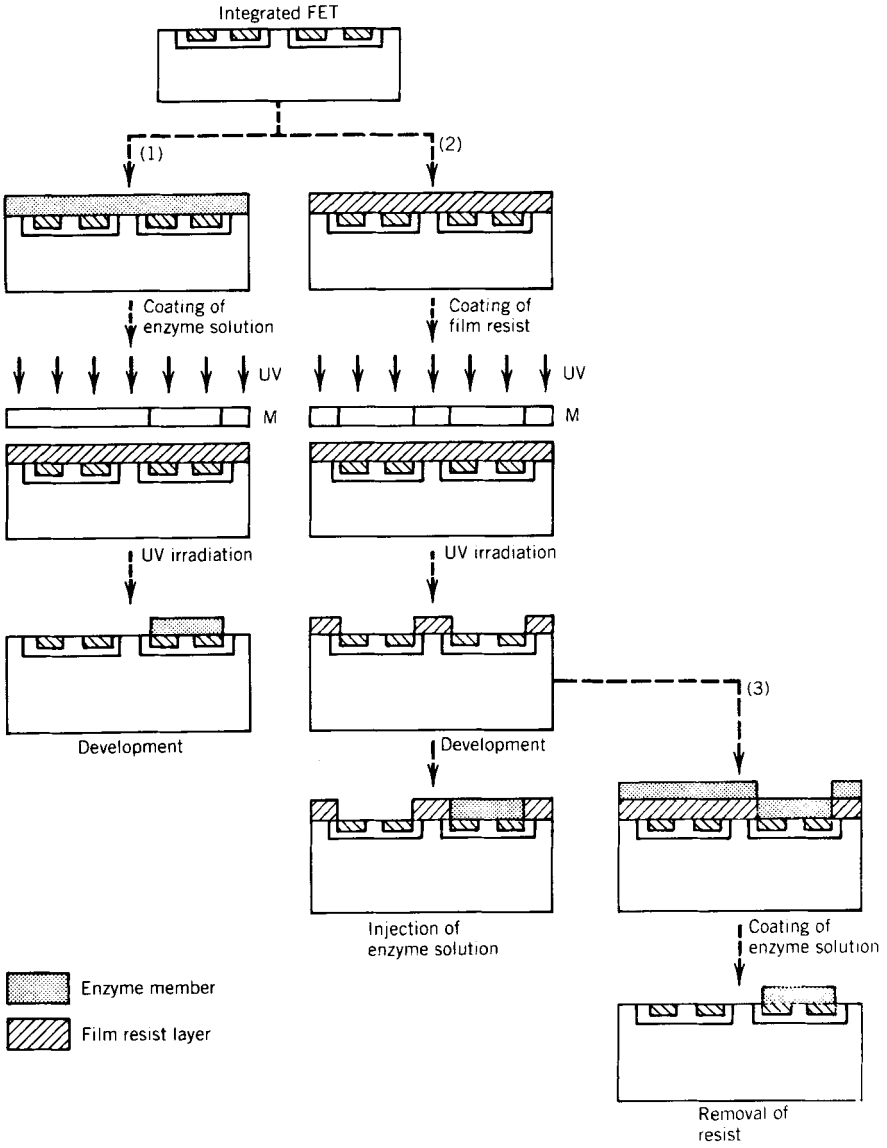

Fig. 8. Flow diagram for constructing a photolithographic enzyme membrane by three methods: (1) direct photolithographic patterning method; (2) micropool injection method; (3) lift-off method. UV, ultraviolet light; M, photomask. (Reproduced by permission of CMC Press, Tokyo, Japan.)

layer is developed using an organic solvent. The injection of an enzyme-immobilizing solution into the micropool formed on the enzyme FET and the curing reaction of the injected enzyme solution yields an enzyme-immobilized membrane on the enzyme FET surface. Recently, an ink jet nozzle originally developed for printing equipment was utilized in combination with the micropool injection method for the injection of an enzyme-immobilizing solution into micropools (46). The ink jet method minimizes the amount of enzyme discarded during enzyme membrane deposition.

3. *Lift-off* (see (3) in Fig. 8) (16, 17). As above, micropools are first formed using a light-sensitive film. An enzyme-immobilizing solution is then spin-coated onto the whole surface of a FET. After completion of the enzyme immobilizing reaction, the FET with the enzyme-immobilized membrane is immersed in an organic solvent to dissolve the cured film layer. The organic solvent immersion process removes the resist layer and the enzyme-immobilized membrane that was coated on top of the resist layer. However, the enzyme-immobilized membrane directly attached on the gate surface of the enzyme FET element remains firmly attached.

The three enzyme membrane deposition methods can be adapted for the preparation of different enzyme-immobilized membranes on a single FET chip simply by repeating the cycle of coating, irradiation, and development procedures in the cases of the first and third methods, and by the injection of a different enzyme-immobilizing solution into a different micropool in the case of the second method.

We have undertaken extensive investigation of the photolithographic patterning method. Three water-soluble photopolymers have been tested: polyvinyl alcohol with stilbazolium pendant groups (6, 8, 20, 47), and two polyvinyl pyrrolidone- (PVP) based photopolymers containing 4,4′-diazidostilbene-2,2′-disulfonic acid (26) or 2,5-bis(4′-azido-2′-sulfobenzal)cyclopentanone (BASC) (7, 9–13) as a photo-crosslinking reagent. Of these three photopolymers, the PVP solution with BASC is better than the other two. Figure 9 is a photograph of a glucose oxidase-immobilized membrane patterned on a FET surface by the direct photolithographic patterning method using PVP with BASC. The photograph shows the enzyme membrane as a transparent rectangle on the right ion-sensitive gate; the left ion-sensitive gate is blank for comparison. The enzyme membrane is 1000 μm long and 200 μm wide. The membrane is 1–2 μm thick.

Three monofunctional FET biosensors sensitive to glucose, urea and triglyceride (7, 9–12, 26), respectively, and two bifunctional ones sensitive to glucose and triglyceride and to glucose and urea (7, 13) were made using the photolithographic patterning method. The performance of these enzyme-modified FETs is described in the next section.

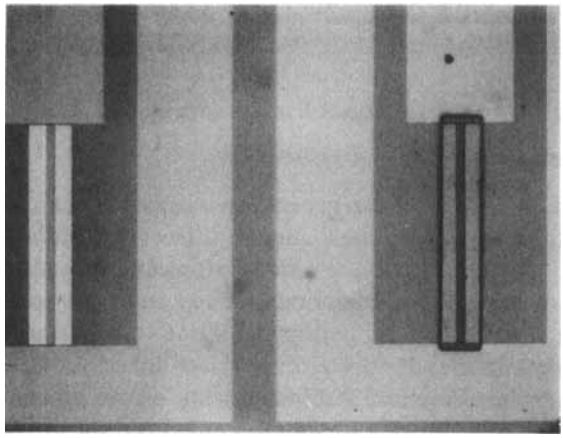

Fig. 9. Photograph of a glucose oxidase-immobilized membrane patterned on an ion-sensitive FET by the direct photolithographic patterning method: enzyme-immobilized membrane (1000 × 200 μm) on right ion-sensitive gate. Left ion-sensitive gate is left blank for comparison.

The micropool injection method was developed by Miyahara et al. (20), and independently by Kimura and his collaborators (15, 16, 43). It was successfully used for a monolithic enzymatically coupled FET, sensitive to urea and glucose, and for the urea-, glucose-, and potassium-sensitive trifunctional FET biosensor (see Section 2.3). The thickness of an enzyme membrane is about 10 μm, and the thickness of an enzyme-immobilized membrane prepared by the ink jet-micropool injection method is 0.1–1 μm. The lift-off method was developed by Kimura and Kuriyama's group and used for the deposition of a urease- and a glucose oxidase-immobilized membrane (16, 17) (see Fig. 8(3)). The enzyme membrane thickness is similar to the thickness of the film resist layer, about 1 μm thick.

Critically comparing the three methods is not possible, because sufficient time has not elapsed since their development to produce data. Information available to date indicates that the direct photolithographic patterning method (see. Fig. 8(1)) is simpler than the other two methods. The number of steps required for construction using the direct photolithographic method is almost half of the number needed when using the micropool injection or lift-off method. Furthermore, the photolithographic method does not require the immobilized enzyme to be in contact with organic solvent which might cause enzyme inactivation. The ink jet micropool-injection method, on the other hand, comsumes less enzyme during preparation of the enzyme-immobilized membrane.

4. PERFORMANCE OF ENZYMATICALLY COUPLED FET WITH PHOTOLITHOGRAPHICALLY PATTERNED ENZYME MEMBRANE

4.1. Monofunctional Enzymatically Coupled FET

4.1.1. GLUCOSE-SENSITIVE FET BIOSENSOR

The optimized procedure for the deposition of a glucose oxidase-immobilized membrane was extensively studied using the direct photolithographic patterning method (9, 10–13). The glucose oxidase-immobilized membrane prepared on a FET surface using photopolymerization to entrap enzyme molecules often peeled off from the FET surface. To improve the adhesive strength of an enzyme-immobilized membrane, the protein was cross-linked with glutaraldehyde in addition to the photoreaction. Adding bovine serum albumin to a glucose oxidase-immobilizing PVP-BASC solution enhanced the cross-linking by glutaraldehyde.

The optimum composition of a glucose oxidase-immobilizing solution (10) is 50 mg of both glucose oxidase and bovine serum albumin in 1 mL of the PVP-BASC solution (10% PVP and 1% BASC in H_2O). Spin-coating the protein solution on a FET, irradiating with UV light through a photomask, and placing the FET in 1–3% glutaraldehyde to cross-link the proteins leaves a well-defined glucose oxidase-immobilized membrane. Enzyme-immobilized membranes prepared in this manner (Fig. 9) adhere well to the FET.

Figure 10 is a plot of the thickness of the membrane as a function of the time of exposure with UV light. Exposure time longer than 2 s gives a patterned membrane of constant thickness of 1.8 μm. Exposing a rectangular-shaped membrane with UV light for longer than 10 s tends to give halation at the edges. Because the response amplitude of the glucose FET sensor is constant over the

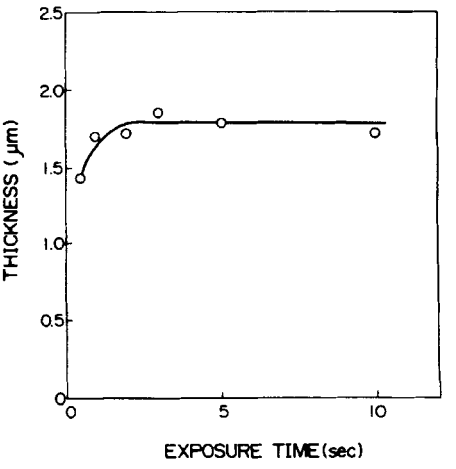

Fig. 10. Effect of UV exposure time on the thickness of a glucose oxidase-immobilized membrane. (Reproduced from Hanazato et al. (10), with permission.)

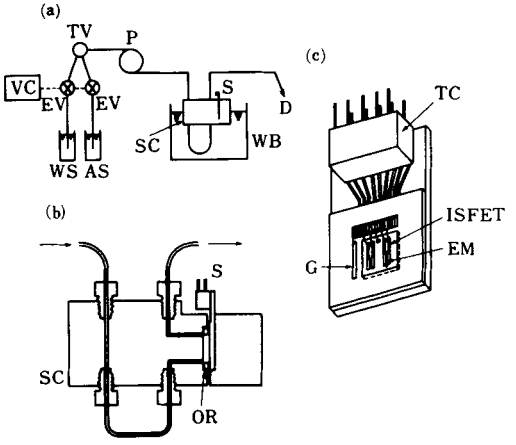

Fig. 11. Schematic diagram of continuous flow apparatus and structure of an enzymatically coupled FET. (*a*) Schematic diagram of continuous flow apparatus: S, enzymatically coupled FET sensor; SC, sensor cell; WB, water bath; D, drainage; P, peristaltic pump; TV, three-way joint; EV, electrical valve; VC, valve controller; WS, washing solution; AS, analyte solution. (*b*) Detailed structure of flow-through cell: OR, rubber O-ring. (*c*) Structure of enzymatically coupled FET (electrical insulation with epoxy resin is not shown here for simplicity): ISFET, ion-sensitive FET; EM, enzyme membrane; G, thin gold film; TC, card edge connector. (Reproduced from Shiono et al. (9), with permission.)

exposure time range from 2 to 10 s, exposure times of 5 s are used for depositing a glucose oxidase membrane on a FET.

A flow-through cell, depicted in Fig. 11, is used to automatically analyze glucose samples (9). Figure 11*a* illustrates the schematic diagram of the continuous flow apparatus, with the flow-through cell depicted in Fig. 11*b*. The inner volume of the flow-through cell is about 0.3 mL. Figure 11*c* shows the structure of the FET biosensor designed for the flow-through apparatus. An integrated ion-sensitive FET chip (ISFET in Fig. 11*c*) with a glucose oxidase-immobilized membrane (EM) on one of the two FET elements. A glucose oxidase-coupled FET sensor (S) is placed in the sensor cell (SC) and the temperature is controlled by a water bath (WB). Phosphate buffer solutions with (AS) and without glucose as substrate (WS) are pumped alternately to the flow-through cell.

Figure 12*a* shows the reponse curves of the glucose sensor with different glucose concentrations, using the flow-through apparatus. The 100% response time is about 15 s over the glucose concentration range from 1.1 to 5.6 mM. Because the intrinsic response time of the FET sensor should be much faster, it was measured by placing the FET sensor in a vigorously stirred 0.02 M phosphate buffer, pH 6.9, and determining the time to achieve full response after an appropriate amount of glucose was added (9, 10). The response curve obtained

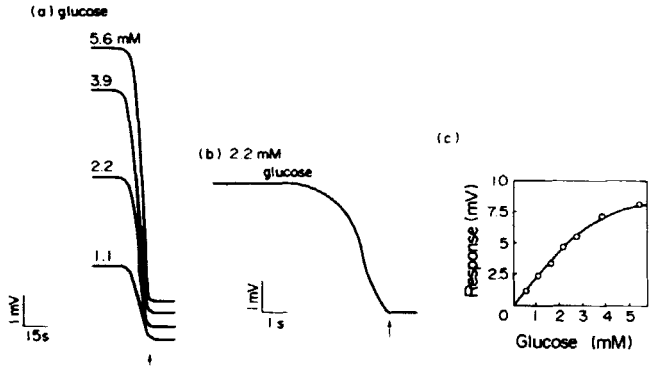

Fig. 12. Response curves and calibration curve of a glucose-sensitive FET sensor. (*a*) Response curves using continuous flow apparatus. An arrow indicates onset of analyte solution flow. (*b*) Response curve under homogeneous condition (see text for details). An arrow indicates addition of glucose. (*c*) Calibration curve using the flow apparatus. (Reproduced from Shiono et al. (9), with permission.)

under the homogeneous conditions is depicted in Fig. 12*b*. The 100% response time is about 4 s and thus is consistent with a glucose oxidase-immobilized membrane of about 1 μm thick.

The calibration curve obtained with the flow-through apparatus is shown in Fig. 12*c*. The output voltage shows linear response to glucose concentration up to 2.2 mM. But it starts to saturate at glucose concentrations exceeding 5 mM. The dissolved oxygen concentration of an analyte solution is expected to influence the response amplitude of the glucose FET sensor, because the glucose oxidase catalyzed oxidation necessitates oxygen as a cosubstrate. The influence of oxygen concentration on the output of the glucose sensor was evaluated (10). Almost the same response amplitudes were obtained up to 3 mM glucose for air- and oxygen-saturated solutions. When glucose concentrations exceeded 3 mM, the responses under oxygen-saturated conditions became larger. Therefore, the responses were restricted when oxygen concentrations were insufficient.

The reproducibility of glucose assays using this sensor was good; the relative standard deviation was less than 2% in the concentration range 0.6–5.6 mM. Figure 13 shows that the glucose-sensitive FET can be used for more than 2000 assays, although the response amplitude decreases gradually after 100 assays and it is about 85% of the initial amplitude after 2000 assays. The detection limit for glucose (signal-to-noise ratio = 3) is 0.05 mM.

We have recently improved the performance of the glucose-sensitive FET using the enzyme gluconolactonase (11, 14). The glucose-sensitive enzymatically coupled FET utilizes the following two reactions:

$$\beta\text{-D-Glucose} + O_2 \xrightarrow{(1)} \text{D-Glucono-}\delta\text{-Lactone} + H_2O_2 \xrightarrow[(2)]{H_2O} \text{D-Gluconic acid}$$

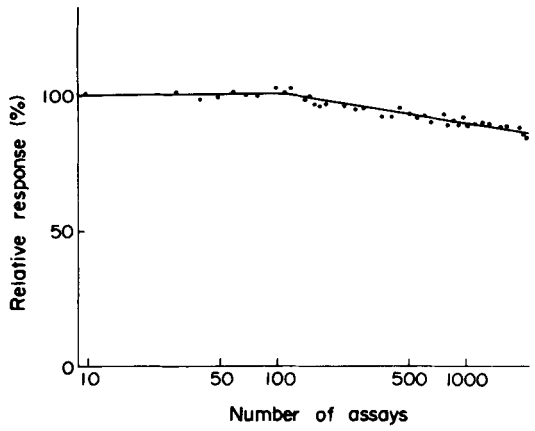

Fig. 13. Long-term stability of a glucose-sensitive FET sensor. A sample solution containing 1.7 mM glucose is used. (Reproduced from Shiono et al. (9), with permission.)

Reaction 1 is catalyzed by glucose oxidase, but the glucose FET requires reaction 2 to produce gluconic acid to give a change in pH. Without gluconolactonase, gluconolactone has to be spontaneously hydrolyzed. However, the spontaneous hydrolysis reaction is slow (11, 14, 22), so the response of the FET with enzyme-immobilized membrane having only glucose oxidase activity would also be slow and small.

Because gluconolactonase is not commercially available, we purified it from crude glucose oxidase to study the role of this enzyme. The effect of the co-immobilization of gluconolactonase and glucose oxidase to form a membrane on the response of a glucose-sensitive FET is shown in Fig. 14 (11). An enzyme membrane containing both enzymes is deposited using the direct photolithographic patterning method (Fig. 8(1)). The upper and lower curves are obtained for the FET with and without co-immobilized gluconolactonase in a glucose oxidase-immobilized membrane, respectively. The importance of incorporating gluconolactonase activity in the enzyme-immobilized membrane is unambiguously shown; the FET with glucose oxidase/gluconolactonase co-immobilized activity shows a large and rapid response, but the FET without gluconolactonase shows no significant response. The optimum response is obtained when the ratio of gluconolactonase activity to glucose oxidase activity exceeds two.

The enzyme-immobilized membrane of our FET biosensor was about 1 μm thick. Gluconolactone produced by the glucose oxidase immobilized in such a thin enzyme membrane is thought to go out of the membrane without being spontaneously hydrolyzed if there is not sufficient gluconolactonase activity in the membrane. If a much thicker enzyme membrane is employed, produced gluconolactone has sufficient resident time for spontaneous hydrolysis before diffusing out of the membrane, which gives a change in pH in the membrane. Therefore, a FET glucose sensor with a thick enzyme membrane is thought to

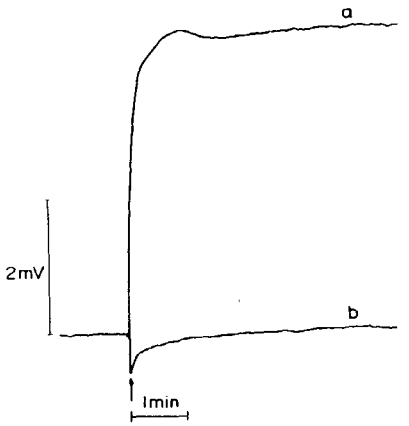

Fig. 14. Response curves of glucose-sensitive FETs for 1 mM glucose: (*a*) enzymatically coupled FET with gluconolactonase/glucose oxidase-co-immobilized membrane; (*b*) enzymatically coupled FET with glucose oxidase-immobilized membrane. (Reproduced from Hanazato et al. (11), with permission.)

give detectable response amplitude, even if the enzyme membrane has no gluconolactonase activity. In fact, some reports of studies in which gluconolactonase was not used suggest that the enzyme-immobilized membrane for a glucose FET sensor should be thick to obtain enough response amplitude and that rapid response could not be expected for such a FET sensor (21, 22).

We also did computer simulation on the effect of the gluconolactonase coimmobilization on the response characteristics of a FET glucose sensor (14). A FET sensor with an enzyme-immobilized membrane of 1 μm thick gives a sub-μV response amplitude without coimmobilizing gluconolactonase. It is also shown that without gluconolactonase an enzyme-immobilized membrane of more than 100 μm is required to give similar response amplitude to our glucose FET.

Some practical applications of a glucose-sensitive FET will be described in Section 4.

4.1.2. UREA-SENSITIVE FET BIOSENSOR

A urea-sensitive FET biosensor made with a urease-immobilized membrane responds to an increase in pH following the decomposition of urea into ammonium ion and carbonate ion (9). Because a PVP-BASC solution containing urease and bovine serum albumin did not give a mechanically stable enzyme membrane, poly-L-lysine instead of bovine serum albumin was added to the urease-immobilizing solution and cross-linked with glutaraldehyde. Optimum performing membranes were obtained when the following solutions were used: PVP-BASC aqueous solution (10% PVP and 1% BASC in water), urease, (75 mg/1 mL PVP-BASC solution), and poly-L-lysine (25 mg/1 mL PVP-BASC solution).

Figures 15*a* and *b* show the response curves of the enzymatically coupled FET sensitive to urea in 0.02 M phosphate buffer at pH 6.9. Using the flow-through cell the 100% response time is about 25 s (Fig. 15*a*), while it is about 6 s under

homogeneous conditions (Fig. 15*b*). The response time is somewhat longer than that of the glucose sensor. The calibration curve depicted in Fig. 15*c* shows that the response amplitude of the urea FET is about one order of magnitude larger than the glucose FET. The urea FET sensor gives linear response up to 5 mM. The relative standard deviation was less than 2% for urea in the concentration range from 1.7 to 16.7 mM. The detection limit (signal-to-noise ratio = 3) was 0.005 mM.

Figure 16 shows the long-term stability of the FET using a 1.7 mM urea solution. The response amplitude of the sensor decreases rapidly after 40 measurements without adding EDTA (ethylendiamine tetraacetic acid) to buffer solutions (Fig. 16, −EDTA). Adding EDTA to the buffer increases the life of the urea sensor. The urea sensor is usable for more than 2000 assays, showing no decrease in the response amplitude (Fig. 16, +EDTA). The rapid decrease in the response amplitude in the absence of EDTA suggests that heavy metal ions inactivate urease. The effect of EDTA on the durability of a urea sensor has been previously reported (48).

The calibration curves of the urea FET are plotted in Fig. 17: the broken line shows the calibration curve for serum urea diluted 10-fold in PBS (see Fig. 17); the solid line is that for pure urea dissolved in PBS. Both curves are linear, but the former has a lower slope than the latter; the ratio of the two slopes is 0.92. The lower slope of the calibration curve for urea in serum samples arises from the

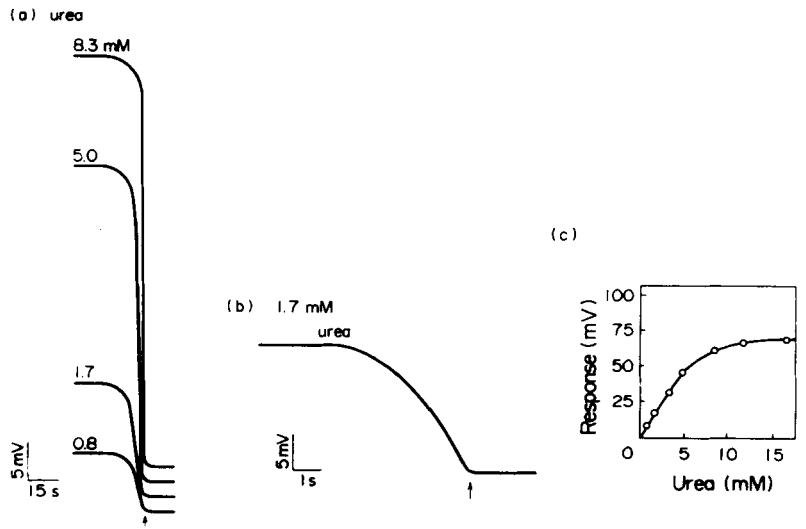

Fig. 15. Response curves and calibration curve of a urea-sensitive FET sensor. (*a*) Response curves using continuous flow apparatus. The arrow indicates onset of analyte solution flow. (*b*) Response curve under homogeneous condition. The arrow indicates addition of urea. (*c*) Calibration curve using the flow apparatus. (Reproduced from Shiono et al. (9), with permission.)

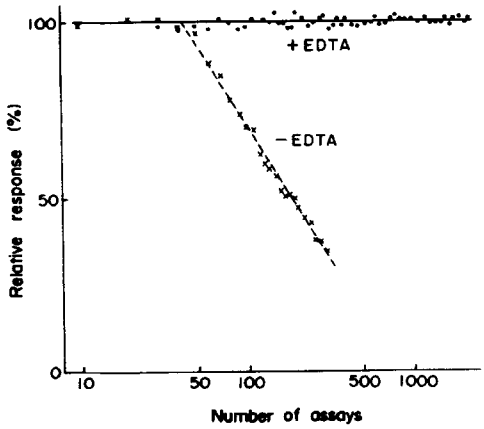

Fig. 16. Long-term stability of a urea-sensitive FET sensor. Phosphate buffer with (—) and without (---) EDTA is used for sample (1.7 mM urea) and wash solutions. (Reproduced from Shiono et al. (9), with permission.)

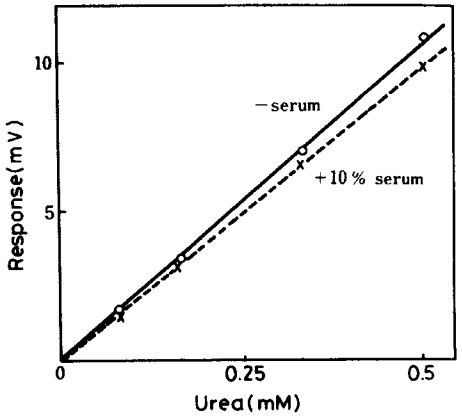

Fig. 17. Calibration curves of a urea-sensitive FET sensor for urea in PBS solutions (Dulbecco phosphate buffer solution without Mg and Ca, pH 7.3) with and without 10% serum. Serum samples are diluted 10-fold with PBS solution. (Reproduced from Shiono et al. (9), with permission.)

larger buffering capacity of PBS with serum compared with PBS without serum (see Section 2.3). Measuring the buffering capacities of PBS with and without 10% serum by titrating the two solutions with HCl shows that their ratio is 0.91 which corresponds well with the slope ratio of the two calibration curves. The reproducibility of the urea FET for serum urea is summarized in Table 2.

4.1.3. TRIGLYCERIDE-SENSITIVE FET BIOSENSOR

Using a lipase-immobilized membrane, the FET biosensor made it possible to analyze for triglycerides (7, 13, 26). Lipase catalyzes the hydrolysis of triglyceride into glycerol and organic acid. In an assay for tributylin the FET biosensor gives

TABLE 2

Reproducibility of Urea-Sensitive FET Sensor

Number	Serum Urea Concentration (mM)	Urea Concentration Determined[a] (mM)	Relative Standard Deviation (%)
1	0.83	0.79 ± 0.01	1.3
2	1.67	1.62 ± 0.02	1.1
3	3.33	3.21 ± 0.03	0.9
4	5.00	5.07 ± 0.07	1.3

[a]Average ± standard deviation of five measurements.

Source: Reproduced from Shiono et al. (9), with permission.

responses up to 0.5 mM, which is in close agreement with its solubility limit in water. In the case of triacetin, the upper limit of the sensor response is near the solubility of triacetin. Triolein, which is minimally soluble in water, cannot be detected by the FET biosensor. Therefore, the immobilized lipase on the FET surface is able to hydrolyze only dissolved triglyceride molecules so that some solubilization technique should be employed to measure triglyceride over a wider concentration range. One preliminary study (26) shows that detergents are able to solve the solubility problem. Of the detergents tested, Triton X-100 is the most effective.

The effect of Triton X-100 concentration on biosensor response is shown in Fig. 18. For 0.4 mM tributylin, which is soluble in water without detergent, there is a drastic decrease in the response with increasing concentration of Triton X-100. In contrast, for 0.4 mM, and 2 mM triolein, which are insoluble without detergent, the biosensor response increases from zero, reaches a maximum value, and then decreases gradually. Figure 18 shows that the sensitivity of the

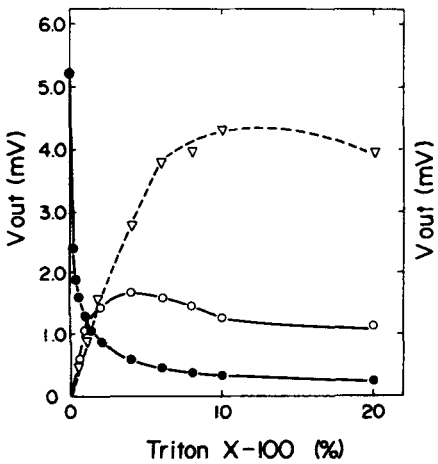

Fig. 18. Effect of Triton X-100 concentration on the response amplitude of a triglyceride-sensitive FET sensor: ●, 0.4 mM tributylin in 10 mM Tris–maleate buffer; ○, 0.4 mM triolein in 1 mM Tris–maleate buffer; ▽, 2 mM triolein in the 1 mM buffer. (Reproduced from Nakako et al. (26), with permission.)

lipase-immobilized FET for 2 mM triolein is about 10 times higher with 10% than with 0.5% Triton X-100 detergent, and is about doubled for 0.4 mM triolein with 10% compared to 0.5% Triton X-100. The higher detergent concentration makes it possible to use the triglyceride-sensitive FET biosensor over a wider concentration range for a variety of insoluble triglycerides. It was found that a 0.4 mM triolein solution became clear with more than 1% Triton X-100 and a 2 mM triolein solution was transparent with more than 6% detergent. Therefore, 10% Triton X-100 was used for further study.

The photopolymer solution used for the immobilization of lipase on a FET contained 10% PVP and 0.3% BASC (7, 13). A lipase-immobilizing solution was prepared by dissolving 150 mg of lipase and 100 mg of bovine serum albumin in 1 mL of the photopolymer solution. A photolithographic lipase membrane was formed on one of two FET elements, similar to the glucose oxidase and urease membranes, except that the development was done in water. After the development, the lipase-immobilized membrane was immersed in a 3% glutaraldehyde solution for the additional chemical cross-linking of the protein molecules.

The performance of the triglyceride-sensitive FET biosensor in 0.001 M Tris-maleate buffer solutions, pH 7.0, containing 10% Triton X-100, is shown in Fig. 19. A linear response is obtained up to 1 mM triolein. The triglyceride FET biosensor is useful for the determination of triolein over the concentration range of 0–3 mM.

4.2. Multifunctional Enzymatically Coupled FET

Two bifunctional enzymatically coupled FETs sensitive to glucose and urea, and to glucose and triglyceride, respectively, using an integrated FET chip with three independently working FET elements (see Fig. 7) were recently described (7, 13). Glucose oxidase/gluconolactonase- and a urease- or a lipase-immobilized membrane were deposited on the right and left FET elements, respectively.

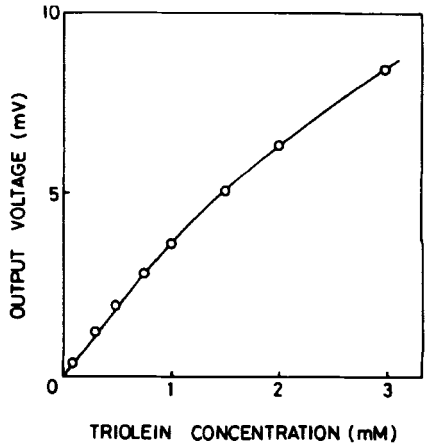

Fig. 19. Calibration plot of triolein concentration against the response amplitude of a triglyceride-sensitive FET sensor. (Reproduced from Hanazato et al. (13), with permission.)

Enzyme membrane patterning procedures were utilized similar to the monofunctional FET biosensors, except that the membranes were developed in water instead of a glutaraldehyde solution. The center FET element was used as a reference FET employing a bovine serum albumini-immobilized membrane. After the three membranes were deposited on the FET surface, they were cross-linked by immersing them in a glutaraldehyde solution to make them mechanically strong.

The response characteristics of the bifunctional FET sensitive to glucose and urea are given in Fig. 20. The upper traces show the response to glucose and the lower ones to urea. The three sets of response curves are for a 3 mM glucose solution (*a*), a solution simultaneously containing 3 mM glucose and 5 mM urea (*b*), and a 5 mM urea solution (*c*). The glucose sensor shows slight interference from the solution containing only urea. Response curves for urea solutions at various concentrations are shown in Fig. 21. The glucose sensor shows no interference from urea <2 mM, and a slight negative response for urea >3 mM.

5. PRACTICAL APPLICATIONS OF ENZYMATICALLY COUPLED FET SENSITIVE TO GLUCOSE

Enzymatically coupled FETs have reached the stage of development where their application to practical sensing problems in various analytical fields is possible. Applications in the field of medical diagnostics are most frequent, although a few are used for the control of bioprocesses, for example, a fermentation bioreactor (12, 49).

The glucose-sensitive FET can be applied to an in vitro blood assay, using a semiautomated flow apparatus (50). Figure 22 shows a schematic illustration of the apparatus. Human blood plasma is sucked up into the sample loop (SL) and then sent to the 30-μL sensor cell (SC) via the mixing coil (MC) and defoaming

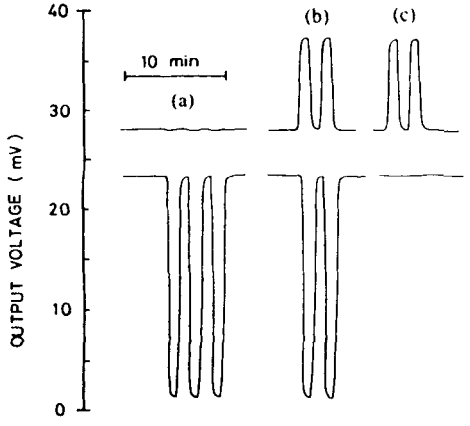

Fig. 20. Simultaneous responses of a bifunctional enzymatically coupled FET for (*a*) 5 mM urea, (*b*) 3 mM glucose and 5 mM urea, and (*c*) 3 mM glucose (upper curves for glucose and lower curves for urea). PIPES–NaOH buffer of 0.01 M at pH 7.0 is used. (Reproduced from Hanazato et al. (13), with permission.)

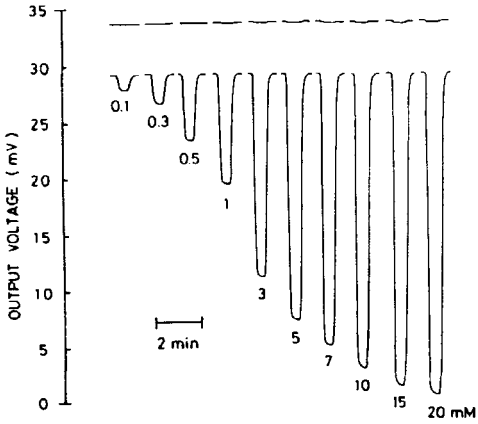

Fig. 21. Response curves of a bifunctional enzymatically coupled FET at various urea concentrations (upper curves for glucose and lower curves for urea). (Reproduced from Hanazato et al. (13), with permission.)

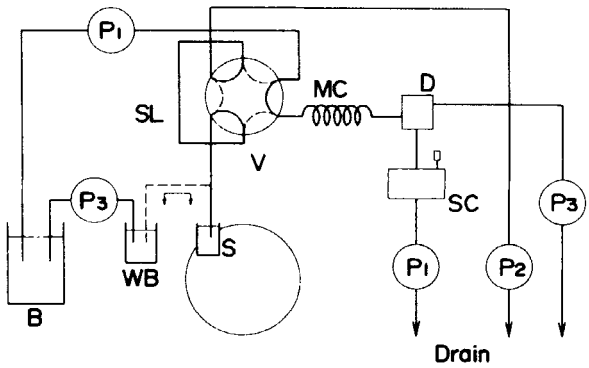

Fig. 22. Schematic structure of flow apparatus: P, pump; SC, sensor cell; SL, sample loop; V, valve; MC, mixing coil; D, deforming device; B and WB, washing solution; S, sample solution. (Reproduced from Nakako et al. (50), with permission.)

device (D) by switching the six-way valve. A monofunctional FET is used that has a co-immobilized glucose oxidase and a gluconolactonase membrane with a linear response for glucose up to 500 mg/dL for 30-μL sample solutions. This sensor was used to analyze a large number of human blood plasma samples whose glucose concentrations had been measured by the conventional enzymatic method. The results of this experimental study are shown in Fig. 23. The glucose concentrations determined by the sensor correlate well (cc = 0.998) with those determined by the enzymatic method.

Taking advantage of the microsized enzymatically coupled FET made it possible for Kimura et al. (51) and Ito et al. (52) to determine glucose in very small samples. A novel blood glucose monitoring system was made. It allowed almost noninvasive glucose monitoring, using transcutaneous effusion fluid instead of

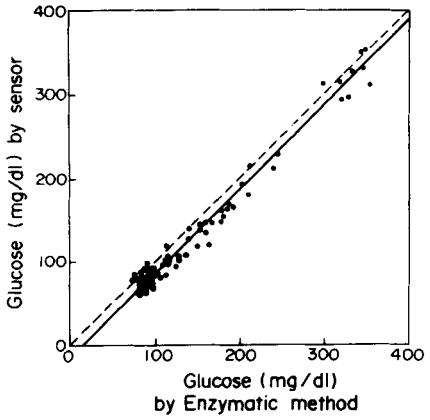

Fig. 23. Relationship between glucose concentrations of human blood plasma samples measured by a glucose-sensitive FET sensor and by a conventional enzymatic method ($Y = 1.007X - 14$, $r = 0.988$, $n = 101$). (Reproduced from Nakako et al. (50), with permission.)

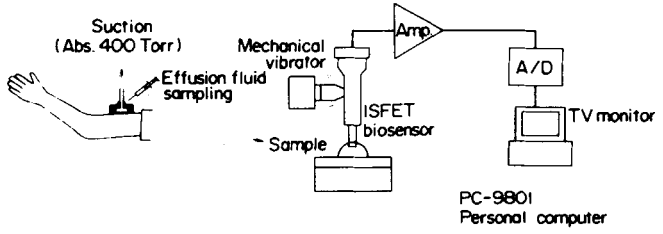

Fig. 24. Experimental setup of a blood glucose monitoring system. ISFET, ion-sensitive FET. (Reproduced from Ito et al. (52), with permission.)

blood. Figure 24 shows the experimental setup of this monitoring system. A small amount of transcutaneous effusion fluid is sucked into the collecting chamber. Then the glucose concentration of a 5-μL sample is determined after diluting to 25 μL with buffer. The results of continuous glucose monitoring by the system are compared in Fig. 25 with glucose concentrations in blood sera taken at the same time. At time = 0, 75 g glucose was loaded orally. The glucose concentration in the transcutaneous fluid after the glucose load measured by the FET system follows almost precisely the time course of the glucose concentration in the blood sera, with about a 10-min delay. This delay is assumed to be due to sampling delay and the effusion delay of fluid passing through the epidermis. It was suggested that the novel monitoring system could be used as a portable blood glucose monitoring device for a diabetic patient.

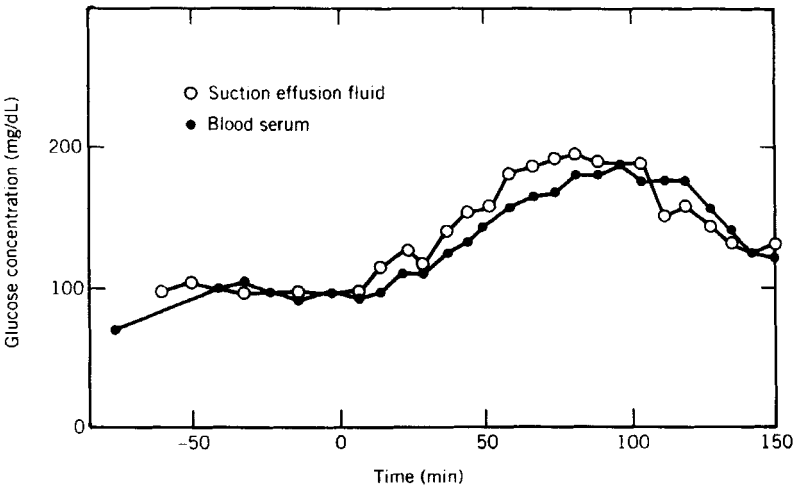

Fig. 25. Comparison of serum glucose and glucose in suction effusion fluid following an oral glucose tolerance test (75 g glucose). (Reproduced from Ito et al. (52), with permission.)

6. CONCLUSION

Methods are now available to make small and well-defined enzyme membranes on FET surfaces. The performance of these enzymatically coupled FETs with a photolithographically patterned enzyme membrane as well as their practical applications are described. It is reasonable to conclude that the enzymatically coupled FETs have reached a stage of development where they can be applied to real-world problems.

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Enzyme-Labeled Antibodies in Bioassays

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1. INTRODUCTION

Antibodies are singularly the most important and widespread type of probe available to biological science. They are capable of recognizing, with different

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affinities, the widest range of molecules (antigens) and molecular structures (epitopes), including lipids, proteins (including other antibodies), carbohydrates, nucleic acids, haptens, and organic compounds, such as organopesticides. The utility of antibodies is improved by our ability to raise second antibody reagents, generate hybridomas secreting monoclonal antibody, and genetically engineer antibody fragments and chimeric fusion proteins (1–7).

Antibodies represent a key component of analytical systems for detecting and measuring a multitude of compounds in a variety of materials and environments. Recognition of antigen (or analyte) by an antibody does not inherently lead to a measurable change unless there is an accompanying aggregation leading to formation of complexes that can be measured by turbidimetry, nephelometry, or visual precipitation. Using antibodies for analyte detection or measurement usually requires that they be labeled with additional detection reagents. Fundamental research, biotechnology process management, clinical diagnosis, and emergent extra laboratory applications, including over-the-counter personal assay kits (e.g., home pregnancy tests), increasingly impose demands for labeled antibodies to fulfill their various requirements. Many methods are now available for labeling antibodies with radioisotopes, luminophors (e.g., fluorescent and luminescent substances) biotin, or enzyme labels, or alternatively detecting antibodies using labeled antibody secondary reagents. Their range of applications has been reviewed recently (8).

The diversity of options available in terms of labels and labeling methods can be overwhelming, making it difficult to select the most suitable for a particular application. Considering the available methods and their relative merits makes it possible to select the best labeling system. The ability to manipulate immunoglobulin and enzyme encoding genes may have a considerable impact on the future design and performance of bioanalytical systems. We will discuss the various options available for enzyme labeling, the extent of enzyme-labeled antibody applications, and personal perceptions concerning their relative merits. Since most assays now require sensitivity in balance with safety, relative simplicity, and a variety of endpoint signals (see below), methods for signal amplification and detection systems are worthy of some discussion. Labeling systems employing radioisotopes and fluorescent or ligand binding compounds will be considered only briefly, since they form the building blocks of some multilayer assays and have been reviewed elsewhere (8, 9).

2. ADVANTAGES AND LIMITATIONS OF ENZYME–ANTIBODY CONJUGATES

The advantages and limitations of enzymes as labels depend on the properties of both the enzyme and the molecule to be labeled. The widespread use of enzymes as antibody labels requires consideration of some aspects of the antibody molecule itself. The availability and characteristics of antibody reagents (native or recombinant) will always be the most important factor controlling assay per-

formance and feasibility. Achieving maximal performance requires careful choice of label, antibody, coupling reaction, and detection method to suit the particular assay. All components must perform well under all conditions. Unusual requirements may be essential in some assays; for example, nucleic acid hybridization reactions require thermostability, resistance to denaturation by organic solvents, and stability at high ionic strength in the presence of chaotrophic agents. The resultant variety and versatility of enzyme–antibody conjugation methods provides conjugates well suited to meet the need for simplicity and/or minimal instrumentation (increasingly to meet the emergent demands of the extralaboratory test marketplace).

2.1. Characteristics Related to Enzymes as Labels

Enzymes are undoubtedly the most widely applied labeling compound (Table 1). Several factors are responsible for this, but all stem from the early recognition that enzymes are biologically and chemically the most active and versatile family of molecules. Their widespread occurrence in all organisms (unicellular and multicellular) means that there is a large repertoire to choose from, each with a broad or narrow range of substrate specificities. This in itself can limit the ultimate utility of enzymes, especially those of broad specificity. For instance, high background activities or interference can result when materials naturally "contaminated" with enzymes or a variety of substrates are assayed. Furthermore, the ubiquity of enzymes in all types of organisms or samples may generate high background signals in bioanalytic assays, especially of body fluids and organism cultures. The need to immobilize enzymes for many industrial or diagnostic purposes generated a wealth of data that enhanced the development of methods and chemistries for labeling enzymes, proteins, and antibodies.

TABLE 1

Enzymes Used as Labels for Antibodies and Other Biomolecules

Adenosine deaminase	Glucosidase
Alkaline phosphatase	Hexokinase
α-Amylase	Horseradish peroxidase
Bacterial luciferase	Invertase
β-Amylase	Lysozyme
β-Galactosidase	Malate dehydrogenase
β-Galactosidase fragment	Microperoxidase
β-Lactamase	6-Phosphofructase
Carbonic anhydrase	Phosphoglucomutase
Catalase	Phospholipase C
Firefly luciferase	Pyruvate kinase
Glucose oxidase	Ribonuclease A
Glucose-6-phosphate dehydrogenase	Urease
	Xanthine oxidase

The breadth of knowledge regarding enzyme structure and function has also led to detailed understanding of their optimal activity rates and the parameters influencing activity. In contrast to fluorescent or radioactive labels where signals are generated rapidly following activation or decay, the signal generated by enzyme conversion of substrate is rapid and controllable: It accumulates and its production can be prolonged as long as substrate and catalyst remain. Individual enzymes can act upon a range of substrates to generate a variety of endpoints either colorimetric, electrochemical, luminescent, or fluorescent. When the endpoint is visible, enzyme labels are appropriate for assays where minimal instrumentation is available or desirable (see below). The flexibility of endpoints also means that a given enzyme conjugate may be applicable in a variety of assay formats and dual analyte assays are also possible without interaction between label and detection methods.

The intensive study of enzymes over the decades has also played a role in establishing their success as probe labels by allowing development of methods to enhance their already significant molecular stability. Many enzymes can remain active after storage in solution at low temperatures or can be lyophilized and reconstituted with no significant loss of activity. Under appropriate storage conditions, enzyme–protein conjugates have long shelf lives, with no harm to the conjugated protein or loss of label activity over time. In comparison, useful radiolabels have relatively short half-lives and the emitted radiation can cause significant damage to the ligand-binding activity and structural integrity of radiolabeled probes (such as proteins). Radiation poses health risks, and the need for repeated labeling makes it difficult to standardize radiolabeled reagents. Therefore, it is difficult to compare results between assays or laboratories. In contrast, enzymes pose no health risks, and can be readily standardized with respect to labeling conditions and activity. This is in part due to the fact that under a given set of conditions, enzymes obey the fundamental Michaelis–Menton equation of activity allowing enzyme:antibody ratios to be determined and standardized. The widespread use of many enzymes in biotechnology on an industrial scale also means that methods are developed for their relatively inexpensive bulk preparation (further aiding standardization) either from natural or recombinant sources. In contrast to other marker molecules, the labeling chemistries required for enzymes can be complicated and expensive where good control of the product is required, while separation of conjugated and unconjugated enzyme can be complex in comparison to other smaller labels which are easily removed. These separation steps are also relatively expensive and may result in poor recovery of label and antibody activity.

Generally, enzymes are relatively large molecules and thus pose several potential problems for their use as labels. First, they have the potential to hinder sterically probe:target recognition or alter the overall functionality of the probe, that is, alter tertiary conformations hindering specific binding and increasing its nonspecific association with other substances. Second, the large size of the enzyme, as compared to radioactive or fluorescent markers, reduces the number of label molecules that can be attached per probe molecule, although this does

not usually significantly impair maximal detection sensitivity since the high turnover rates of enzymes more than compensate for this fact. Third, due to their relatively large size, probes labeled with enzymes may behave differently from the native unlabeled probe. Binding may be hindered kinetically due to slower diffusion, while other properties of the probe such as its permeability through membranes, migration through different support matrices or electrophoretic mobility will also be altered.

Last, while the large size of enzymes increases the variety and number of available covalent linkage sites outside the critical active site, too many linkage sites can be problematic. Multiple cross-linking of probe and label molecules must be carefully controlled so that the probe and enzyme active sites remain functionally unaltered while achieving the optimum label to probe ratio. To maintain consistent assay performance the conjugate size distribution must be controlled as well as the average number of molecules coupled. Varying proportions of macromolecular complexes may be produced during coupling reactions or in storage and this can alter probe performance and nonspecific binding during the assay procedure. The need to closely control conjugate composition may limit the coupling chemistries that can be effectively utilized with a particular enzyme. The availability of reversible (or irreversible) enzyme inhibitors and activators can be exploited to improve stability of conjugate activity and to minimize or prevent interference from enzymes contained in the sample. Alternatively, the known or suspected presence of an activator or inhibitor in samples may restrict enzyme applicability for some analytical procedures. This is an important consideration in competitive assays or methods whereby the receptor–ligand interaction may be required to induce a modulation in enzymic activity (see below).

2.2. Characteristics Related to the Structure and Properties of Antibodies

Antibodies are glycoproteins composed of two identical heavy and light chains held together by disulfide bonds and hydrophobic interactions and stabilized by branched (N-linked) and linear (O-linked) carbohydrate chains. The protein backbone has exposed terminal amino (especially the epsilon-amino group of lysine residues) and carboxyl groups as well as hydrophobic side chains and aromatic groups. Although the thiol groups are normally present as disulfide bonds stabilizing the molecule, partial reduction makes a few available for reaction without causing too great a loss of stability. The carbohydrate moieties are potential targets for binding of secondary labeling reagents such as lectins, and specific reactive groups may also be made available by partial oxidation to yield potential covalent coupling sites (10). It is also possible that hydrophobic sites may be utilized, either directly for attachment or indirectly to stabilize conjugates. These structural features each represent potential and demonstrated sites for the specific attachment of enzymes onto antibody molecules, although each labeling chemistry can impose individual restrictions upon end utilization (see below). The domain structure of antibodies with individually and com-

pound prescribed functions, makes it possible to utilize broadly specific binding properties to indirectly attach labels using second antibodies (anti-species, anti-class, or anti-subclass), or bacterial immunoglobulin-binding proteins (protein A, protein G). In these cases a single labeled secondary reagent may be usable with a large number of primary probes in separate assays, simplifying assay development and conjugate quality control. Many of the antibody domains are encoded by individual exons in the genome, which makes them suitable as targets for genetic engineering (see below).

The large number of potentially available reactive groups and their random distribution over the surface of an antibody complicates their utilization as enzyme attachment sites. Specific structures along the length of antibody molecules serve a variety of effector functions apart from specific sites or paratopes for binding to antigen (11). Antigen binding sites must remain unaffected by the attachment of an enzyme moiety both during the process of attachment (i.e., without allowing denaturation of the tertiary structure) or following attachment (i.e., without sterically blocking subsequent antigen binding). Conjugate stability may also require minimal chemical modification of exposed reactive sites not involved in enzyme attachment. Local alterations in charge or hydrophobicity will result in altered tertiary structure and may adversely effect solubility and interaction with other protein molecules. Similarly exposed thiol groups or oxidized carbohydrate chains produced during coupling may require blocking to avoid slow covalent polymerization of conjugates during storage. In some circumstances, it is desirable that structures not involved in antigen recognition and binding remain accessible, such as sites recognized by effector molecules, receptors, and second reagents (e.g., lectins, species-specific antisera). To preserve such structures, incomplete usage of available conjugation sites is required, decreasing the maximal label incorporation and sensitivity achievable. This is further compounded by the relatively large size of enzymes although increased knowledge and ability to engineer enzyme active sites may reduce the importance of this factor.

2.3. Influence of Antibody Production Technique

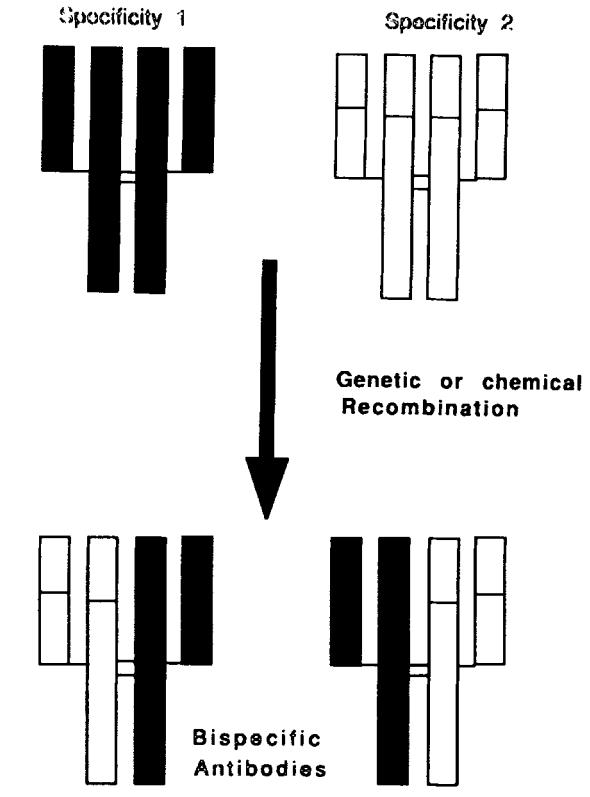
The production of hybridomas secreting a single species of (monoclonal) antibody (12) was the primary step leading to the present proliferation of available antibody-enzyme conjugates and their utilization. Until this time, enzyme-antibody conjugates were generated using polyclonal antisera. However, as diagnostic and research tools, they were generally unsatisfactory. The diverse range of specificities of polyclonal probe reagents impairs discrimination between related but dissimilar analytes. Highly specific polyclonal reagents require costly and complex purification processes for adsorbing undesirable specificities or cross-reactivities, with poor recovery rates and reproducibility. The majority of immunoglobulin molecules contained in even a very high titer polyclonal antiserum are capable of binding to the immunogen under any conditions, and although affinity purification can isolate these, the yield is poor and

binding properties of such preparations may be significantly impaired in comparison to the original polyclonal antiserum. Furthermore, due to the diversity of the antibody response to immunogen, only a small fraction of the analyte-specific antibody population contained in a polyclonal antiserum may be involved in recognition of the analyte at the desired concentration under assay conditions (i.e., steric, conformational, and affinity influences may allow binding of antibodies to only a few of the potentially recognizable epitopes). Labeling of polyclonal antisera in general is therefore inefficient in terms of specific activity of reagent:enzyme coupled and wasteful of reagents, often requiring higher reagent concentrations than monoclonal reagents to achieve similar detection sensitivities. These problems become particularly important where a complex, costly labeling reaction is required. Standardization of polyclonal reagents, even before enzyme labeling, is therefore difficult and such reagents are generally poorly suited for accurate quantitative assays unless their stability allows them to be prepared on a very large scale.

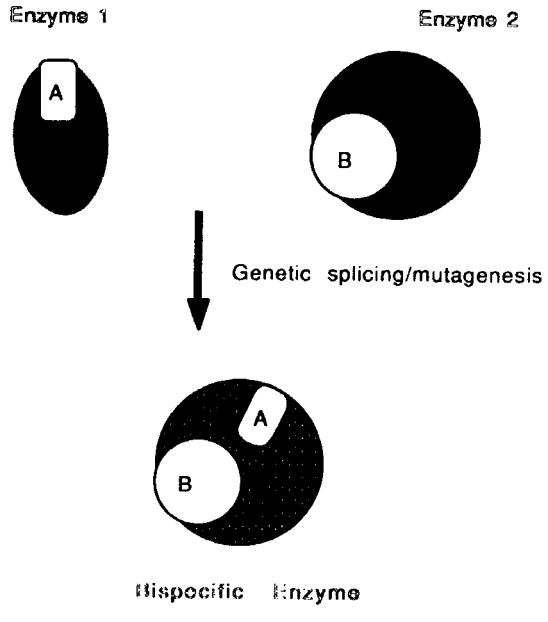
Despite the inherent problems outlined above, the fact that polyclonal antibody reagents are used as enzyme-labeled probes testifies to the fact that these reagents have some valuable characteristics. First, they are relatively easily and rapidly generated in significant quantities. Furthermore, it is relatively easy to produce species-specific antisera. Second, they have a generally higher affinity for an analyte than monoclonal antibody reagents. Third, although possibly less true in the future, they are usually least expensive as bulk antibody reagents. Fourth, where the analyte has multiple isoforms, a monospecific antibody may be incapable of identifying all forms and may not result in desirable assay performance. These characteristics, individually or combined, are essential for some applications.

Increasingly, the advantages of two-site immunoassays in terms of sensitivity and specificity, and the use of enzyme-labeled antibodies in vivo means that monoclonal antibodies are preferred for detection of analytes (8). It is also easier to generate many of the desired specificities to individual structurally related haptens or organic chemical compounds and idiotopes expressed on other antibody molecules. Where assay cross-reaction with multiple isoforms is required, the specificity of monoclonal antibodies can be used to advantage by using a closely controlled mixture of labeled monospecific antibodies to create an ideal multiclonal antiserum with precisely tailored assay characteristics. Once a hybridoma cell line or recombinant antibody clone has been generated, it is possible to maintain a perpetual and consistent supply derived from mammalian, plant, or microorganism cultures (2, 13–17).

Monoclonal antibody technology opens up a potential for modifying antibodies to better suit immunoassays. Chimeric antibodies with dual specificity are already available (18) (see Fig. 1). Half of the antibody derives from an anti-analyte cell line, the other from anti-label. Such antibodies will cross-link antigen and the labeling enzyme from relatively crude mixtures and no covalent labeling is required (19). Murine monoclonals can be humanized by grafting the genes for the binding sites of a monoclonal antibody into the appropriate part of the



(*a*)



(*b*)

Fig. 1. Schematic view of the principles of formation of (*a*) bispecific enzymes and (*b*) bispecific antibodies.

gene for a human immunoglobulin molecule. The resulting molecule is essentially of human origin but possesses the binding properties of the murine immunoglobulin (20). Immunoassays using such antibodies should be less prone to interference by heterophilic antibodies. Extension of this gene-grafting technique makes it possible to alter the specificity and avidity of existing monoclonal antibodies and could be used to create labeled antibodies by grafting together enzyme and immunoglobulin genes (see Fig. 2). None of these possibilities could be achieved for polyclonal antisera.

A further advantage of monoclonal antibody technology is the ability to generate otherwise impossible antigen specificities via in vitro immunization. Many desirable immunogens are insoluble, weakly immunogenic, too toxic to be injected into animals in large quantities, or very rapidly eliminated from the circulation. Antibodies with these specificities are impossible or very costly to produce using live animals and are of poor quality. Antigenic stimulation of spleen cells in tissue culture stimulates the proliferation of the clones that will produce the appropriate antibody populations. Subsequent hybridization and cloning of these cell lines allows production of monoclonal antibodies with the desired specificity (21). Similarly, the spleen cells from in vivo immunized animals can be cultured in the presence of high antigen concentrations prior to hybridization to improve the probability of cloning desired cell lines. These techniques could also be extended to produce human monoclonal antibodies for use in immunoassay or therapy by stimulating cell lines derived from surgically excised spleen, tonsil, or peripheral blood from normal or affected individuals, which therefore increases the probability of cloning them.

3. TYPES OF ENZYME LABELS

A combination of considerations including flexibility (endpoints available), cost (availability and suitable coupling procedures), tradition (why use a different enzyme that can do the same as the proven existent enzyme label), detection sensitivity, stability, safety of detection reactions, and availability of coupling methods determine those enzymes currently utilized as labels. Given the many points above in favor and against a particular enzyme being suitable for probe labeling, there are indeed relatively few enzymes that predominate as labels for antibodies (8). Genetic engineering, however, has provided alternative sources and types of enzymes available with an increased range of natural or modified activities. Our discussion will focus on those enzymes presently used for labeling antibodies and a consideration of the activities of their various isoforms or sources (natural sources as opposed to commercial sources).

3.1. Peroxidases

Peroxidases are isolated from a wide variety of sources and many are used as labels. The most common peroxidases are from plant tissues, including turnip,

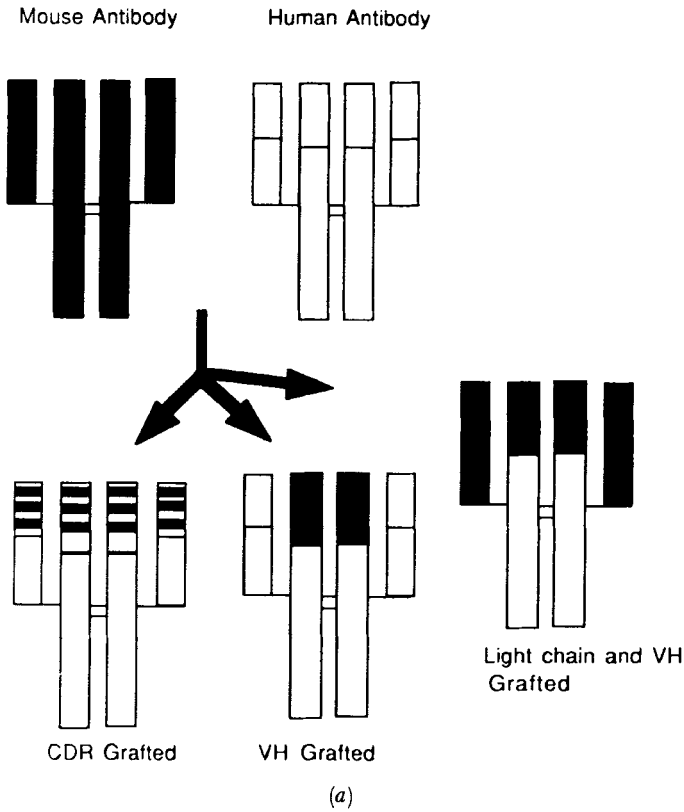


Fig. 2. Schematic view of some types of genetically engineered antibodies: (*a*) chimeras formed between different species antibody genes (i.e., humanization); (*b*) examples of genetically engineered fragments and possible attachment points for enzymes (genetically or chemically). Fv fragments are generated by (i) incorporating a linker peptide between domains, (ii) creating sites for intrachain disulfide bonding, or (iii) unstable formation of Fv by random association of domains (via hydrophobic patch interactions).

Japanese radish, and the root of the horseradish. The enzyme from horseradish is probably the most widely used enzyme label.

Horseradish peroxidase (HRP) is a hemoprotein with extensive glycosylation. A large number of isoenzyme forms have been identified (22, 23) which differ in amino acid composition, molecular weight, and extent of glycosylation. The substrate specificities, pH optima, and reaction kinetics of the groups of isoenzymes are also different (24). The simplest classification is as acidic, neutral, or basic, depending on their electrophoretic mobility at near neutral pH. The distribution of isoenzymes varies with the tissue source, basic isoenzymes being responsible for the majority of activity found in the horseradish root. The main basic isoenzyme has a molecular weight of about 40 000 and has a broad pH

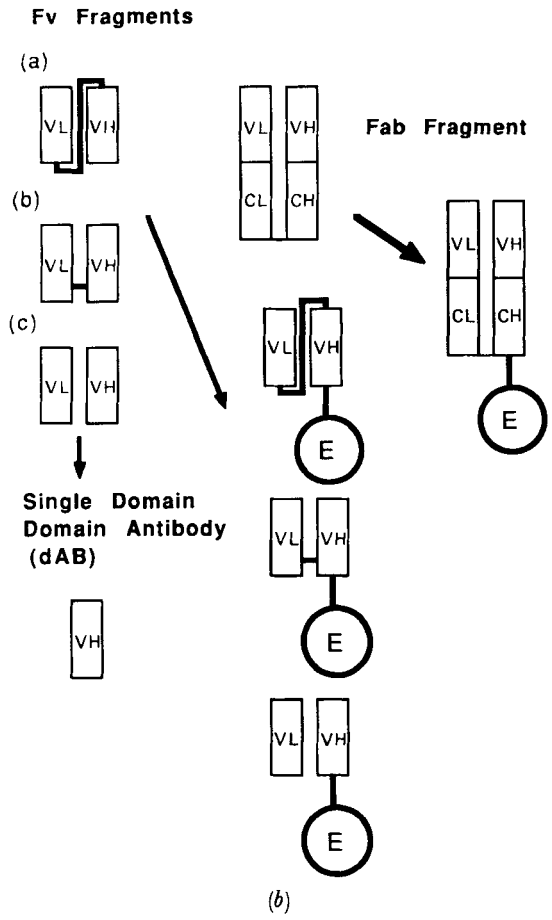


Fig. 2(b)

optimum between 6 and 7 with typical substrates. Commercially available isoenzymes have eight branching carbohydrate chains and only two available lysine side chains. Specific activity is about 4500 U/mg.

Peroxidase catalyzes the oxidation of a wide range of substrates, including ascorbate, ferrocyanide, cytochrome C, and many organic molecules, for example, the leuco form of many dyes. Several of the more sensitive detection reactions have the disadvantage of using potentially carcinogenic substrates (benzidine derivatives, *o*-phenylenediamine) and therefore the reagents are often presented as tablets that can be handled safely. HRP can use several oxidants, including hydrogen peroxide, organic peroxides (e.g., urea peroxide, MeOOH, EtOOH), perborate ions, and superoxide radicals. It requires two electron

equivalents to recycle back to the original enzyme state. The heme group is essential for activity and undergoes spectroscopic changes during the reaction indicating that iron atom participates in the reaction mechanism. The oxidation reaction is thought to proceed in two stages via a free radical process. Each step transfers a single electron and may involve different substrate molecules. The overall reaction can be represented as follows:

$$\mathrm{HRP} + \mathrm{H_2O_2} \longrightarrow \underset{\text{(Compound I)}}{\mathrm{HRP^{**}}}$$

$$\underset{\text{(Substrate)}}{\mathrm{AH_2}} + \mathrm{HRP^{**}} \longrightarrow \underset{\text{(Oxidized product)}}{\mathrm{AH}\cdot} + \underset{\text{(Compound II)}}{\mathrm{HRP^{*}}}$$

$$\mathrm{AH_2} + \mathrm{HRP^{*}} \longrightarrow \mathrm{AH}\cdot + \mathrm{HRP}$$

Its utility as an enzyme label is due in part to its relatively good stability characteristics (as a lyophilized powder it may be stored for years at 4 °C and as a purified aqueous solution it can retain undiminished activity for over 12 months at 4 °C). HRP has a high specific activity and broad substrate specificity. The iron atom within the active site has a single free molecular orbital available for substrate and oxidant interaction; hence, activity can be reversibly inhibited by alternative ligands such as cyanide and sulfide at concentrations of about 10^{-5} M.

HRP can be attached to antibodies by a large variety of reactions. Coupling is possible via carbohydrate, amino, and carboxylic acid side chains. The products are usually purified by gel filtration to separate free HRP (40 kDa), free antibody (140–200 kDa), and conjugate (>180 kDa). HRP is reversibly bound by the lectin Concanavalin A, which can be used in conjunction with protein A specific isolation of conjugates from reaction mixtures, because only conjugated molecules will be retained by both columns (25). This minimizes nonspecific binding and guarantees that every bound antibody molecule is capable of contributing to the signal from an assay.

Antibodies have been raised to HRP and used to construct peroxidase-antiperoxidase layers in assays to increase signals and thus sensitivities. This is particularly useful where high background signals are expected, because the signal-to-noise ratio can be increased many-fold (27).

Several enzymes with peroxidase-like action have also been used in immunoassays. Microperoxidases are catalytically active fragments obtained from cytochrome c by proteolytic action. They consist of the heme group covalently coupled to a short peptide alpha helix (26). The active site structure is similar to that of peroxidase: Four of the six possible coordination bonds of the iron atom are occupied by bonding to the porphyrin while the fifth complexes with a histidine residue and the sixth is exposed to the environment and forms the catalytically active portion of the molecule. The reaction mechanism and spectrum of substrates is similar to HRP, although the specific activity is variable

according to the substrate used. Differing lengths of peptide chain arise, depending on the proteases used to prepare the microperoxidase. 6-MP (28), 8-MP (29), and 11-MP (29) have all been used as labels in immunoassays. Although the heme environment and hence catalytic activity is constant, the microperoxidases have different potential coupling sites. In particular, the 6 and 8 amino acid peptides have a single amino group and only one carboxyl group, offering improved control over covalent linking to other molecules. A number of coupling methods are used with microperoxidase, including two-step activation reagents (29, 31), and heterobifunctional reagents (28, 32).

Microperoxidase–immunoglobulin conjugates are mainly used in the histochemical localization of antigens, although 8-MP labeling is used in solid phase immunoassays with colorimetric (32) and chemiluminescent (28) endpoints. These are small labels and are suited for controllable covalent attachment to proteins because they have only a few reactive groups. However, they tend to bind via hydrophobic interactions and high nonspecific binding can limit the sensitivity of assays.

Catalase is also capable of performing peroxidase-like reactions. It has a high specific activity (40 000 U/mg), but most of the substrates require detection in the ultraviolet and hence are impracticable for immunoassay use.

3.2. Alkaline Phosphatase

Alkaline phosphatase (AP) is an orthophosphoric-monoester phosphohydrolase with an alkaline optima (pH 8.0–10.5), whereas acid phosphatases have activity optima at pH values below 7.0 (generally 4.0–5.5). Alkaline phosphatases are isolated from a variety of organisms and tissues. They exhibit significant heterogeneity, often with organ- and tissue-specific isoforms. These isoforms are believed to arise from post-translational modifications (in *Escherichia coli*) or from genetic polymorphism. For example, human placental AP is encoded by three alleles giving rise to 6 isozymes. This facet of human AP has been successfully exploited as a marker of certain disease states, such as some forms of cancer, where serum levels increase. Other sources of AP include calf and chicken intestine or as prokaryote expressed recombinant AP. The specific activity of the enzyme from calf intestinal mucosa is high (approximately 1000 U/mg). Calf intestinal mucosa is the preferred source.

Alkaline phosphatases are typically dimeric zinc metalloenzymes ranging in size from 80 to 145 kDa. They catalyze a nonspecific phosphomonoesterase reaction of the following type:

$$\text{Orthophosphoric monoester} + H_2O \longrightarrow \text{Alcohol} + H_3PO_4$$

Their ability to remove terminal monoesterified phosphate from both DNA and RNA is unaffected by the base, the position of the phosphoryl group, or the sugar moiety, and this makes them particularly valuable as molecular biology reagents. Calf intestinal AP also catalyzes hydrolysis of phosphate esters of primary and secondary alcohols, sugar and cyclic alcohols, phenols, and amines. Zn^{2+} is

essential for activity and maintenance of the structure of most APs, although Mg^{2+} ions also aid heat stability and activity. Co^{2+} will substitute for Zn^{2+} in human placental and *E. coli* AP. Most forms are irreversibly inactivated by pH values below 2.3–4.0 and their activity can be inhibited by chelating agents (e.g., 1 mM cysteine, EDTA, *o*-phenanthroline), inorganic phosphates (*E. coli* AP), and L-phenylalanine (human placental AP). Several special substrates have been devised making it possible to use spectrophotometric, fluorescent, or chemiluminescent endpoints to detect femtomole or attomole quantities (33, 34).

Storage characteristics of the native enzyme are generally good, with activities maintained over years. Alkaline phosphatase conjugates are usually prepared via amino or carboxylic acid side chains and purified by gel filtration chromatography. Conjugates are very stable, but the enzyme is costly due to the limited supply of calf intestine. Alkaline (and also neutral and acid) phosphatase enzymes in biological samples are a potential problem with the use of this enzyme label. Careful washing of solid phases may be required to ensure no interference in assays.

3.3. Glucose Oxidase

Glucose oxidase (GO) from *Aspergillus niger* is a flavoenzyme that has been used since 1956 to determine glucose (e.g., glucose dipsticks) (for review, see Libeer (35)). Glucose oxidase catalyzes the oxidation of D-glucose to generate H_2O_2 in the general reaction

$$\beta\text{-D-Glucose} + \text{Enzyme-FAD} \longrightarrow \text{Enzyme-FADH}_2 + \beta\text{-D-Gluconolactone}$$

$$\text{Enzyme-FADH}_2 + O_2 \longrightarrow \text{Enzyme-FAD} + H_2O_2$$

This enzyme is often employed in assays coupled to peroxidase and a chromogen for generation of a colorimetric signal or for the production of luminescence in the presence of luminol. GO is a dimeric enzyme (160–186 kDa) with each subunit containing 1 mol Fe and 1 mol FAD held together by disulfide bonds. It is a very stable molecule with a broad activity pH optimum ranging from 4.0 to 7.0. It is inhibited by Ag^+, Hg^{2+}, or Cu^{2+} ions. The specific activity is relatively low (200 U/mg) but mamalian biological fluids do not contain enzymes that carry out a similar reaction; hence, interfering enzyme action is not a problem in separation or homogeneous assays. Conjugates are usually prepared via amino or carboxylic acid groups and purified by gel filtration.

3.4. β-Galactosidase

β-Galactosidase (also known as lactase) is a useful enzyme marker both in bioassays and for genetic recombination experiments. Although it has a widespread distribution among organisms, the *E. coli* form predominates as an antibody label. It catalyzes the general reaction

$$\beta\text{-D-Galactoside} + H_2O \longrightarrow \text{D-Galactose} + \text{Alcohol}$$

E. coli β-galactosidase is a 540-kDa tetrameric enzyme with each subunit having an active site. It is often believed to be present in association with another protein, giving additional stability and enhancing activity. Considerable study has been invested in methods for its immobilization. It has a pH optima of 6.8, which is identical to that from *Aspergillus oryzae* (100 kDa), while bovine testes β-galactosidase (68 kDa) has optima between pH 4.3 and 4.7. This latter form also hydrolyses galactose 1 → 3 linkages in glycoconjugates. *E. coli* β-galactosidase activity is stimulated by monovalent cations and 5% methanol, ethanol, isopropanol, and *n*-propanol, which increase the rate of *o*-nitrophenyl and β-D-galactopyranoside cleavage. It requires Mg^{2+} ions and β-mercaptoethanol for optimum activity.

β-galactosidase has a moderate specific activity (600 U/mg) and hydrolyzes a variety of substrates, providing colorimetric and fluorescent signals. The high sensitivity of detecting the enzyme product (β-galactosidase is among the enzymes with the highest detection sensitivity, attomole quantities or better) and the enzyme stability more than compensate for the specific activity deficit. Conjugates are often produced via the free thiol groups that are present on the enzyme surface. This has the advantage of allowing the use of highly controllable two-step coupling reactions without damaging enzyme activity. The ease by which this enzyme can be cloned will likely increase its use in conjugates. As part of the bacterial *Lac* operon, the gene sequence and the control of biosynthesis are well understood, making genetic engineering of the molecule relatively simple.

3.5. Urease

Urease (urea amidohydolase) occurs in many organisms, especially bacteria, yeasts, and plants. The best sources are Jack beans (*Canavalia ensiformis*), the commonest, and *Bacillus pasteurii*. Urease catalyzes the hydrolysis of urea in the general reaction

$$(NH_2)_2CO + 3H_2O \longrightarrow CO_2 + 2NH_4OH$$

Ammonium carbamate is formed in citrate or Tris buffer. It is a 480-kDa protein with a pH optimum of 6.0. The enzyme is very specific for urea or hydroxyurea. It is inhibited by heavy metals, while it is stabilized by 1×10^{-3} M EDTA. The specific activity of urease is very high (10000 U/mg), but the potential detection sensitivity is lost because the reaction products are difficult to detect. Urease conjugates because of free thiols are not particularly stable. Urease is important in noninstrumented assays because activity can be visualized via pH-sensitive dyes.

3.6. Luciferases

Luciferases are a diverse group of enzymes that catalyze bioluminescent reactions. The enzyme and reaction are broadly the same in all bacterial systems studied, but other organisms produce different enzymes that require different substrates. Bacterial and firefly luciferases are used as labels and are also impor-

tant as indicator reactions in the measurement of other enzymes via production or consumption of adenosine triphosphate (ATP), flavine mononucleotide ($FMNH_2$), NADH, or NADPH.

Bacterial luciferase catalyzes the oxidation of reduced flavin mononucleotide and a long-chain aldehyde by molecular oxygen to yield FMN, the corresponding acid, water, and light in the general reaction

$$FMNH_2 + RCHO + H_2O \longrightarrow FMN + RCO_2H + H_2O + \text{Light}$$

Naturally occurring luciferase is a dimeric protein of about 80 kDa composed of identically sized subunits (alpha and beta). It has a single reduced flavin binding site with a sulfhydryl group near its reaction center. It has a pH optimum around 6.8 and can be inhibited by oxidase inhibitors (e.g., 3,2-diethylaminoethyl-2,2-diphenyl valerate at low concentrations (10^{-5} M), riboflavin, and *p*-chloromercuribenzoic acid).

Firefly luciferase catalyzes the reaction of adenosine triphosphate and a substituted benzothiazole luciferin. The quantum yield of the reaction is high and therefore measurement of the enzyme offers good sensitivity. Coupling of luciferase to other molecules is a problem because there is a highly reactive amino group in the active site that must be protected from reaction with coupling reagents (36, 37).

The luciferase isolated from the burrowing clam *Pholas dactylus* is essentially a peroxidase, which oxidizes a complex organic side chain on the surface of a luciferin. This enzyme has not been used in imunoassays; however, the luciferin is a substrate for horseradish peroxidase, which produces a very sensitive assay for that enzyme (38).

4. LABELING REACTIONS

Labeling reactions can be broadly divided into those employing specific or nonspecific linkages (39, 40). Therefore, we will not review labeling reactions, but will instead outline the principal considerations salient to the use of enzyme-antibody conjugates in bioanalytical systems.

Three methods of conjugation are most widely used: (1) direct chemical coupling, (2) unlabeled or immunologic conjugation where enzyme and antibody are linked by an anti-enzyme antibody, and (3) the use of linking molecules where enzyme and antibody are linked via an auxillary molecule. The first method is most widely used, but suffers from a number of disdavantages, such as cost, complexity, poor control of conjugation rates and substitution levels, and increased loss of activity of the conjugate components. The second method is used in many assays, particularly immunocytochemical methods and for signal amplification, although the complexity and timing of such methods is greater than desirable. The last method is also restricted in application, since it most commonly requires biotinylation of both enzyme and antibody with avidin or streptavidin used as a linking reagent.

The choice of a labeling strategy depends largely on the format of the assay in which the conjugate is applied. High levels of conjugation are often required by immunoassays where the size of enzyme:antibody conjugate is relatively unimportant but sensitivity is, while low-level substitutions (1:1 enzyme:antibody) are preferred for immunohistochemistry assays where permeability of the conjugate is of paramount importance.

Many of the original methods of chemical coupling are still applied despite their disadvantages, because the development of homo- and heterobifunctional reagents has increased their utility by overcoming the problems of loss of biological activity and substitution rates. In most situations the chemistries utilized are the same as those applied to the immobilization of enzymes (see Chapters 1 and 3).

Perhaps the most widely used (and least controlled) procedure involves the use of glutaraldehyde (GA) to form a linkage between ε-amino groups, principally of lysine residues (41). This method suffers from the usual problems of high polymerization, formation of homo- and hetero-coupled molecules and loss of biological activity. However, it is a relatively simple, cheap, and effective method for a wide range of enzymes and proteins. Two-stage reactions have been developed (42), which give better labeling with less biological inactivation, but they are still wasteful of enzyme and antibody. The degree of conjugation and cross-linking depends on the molecular form of glutaraldehyde used. Monomeric glutaraldehyde (e.g., electron microscopy grades) polymerizes rapidly at mildly alkaline pH, but can be used in two-step reactions. It tends to produce a conjugate with lower molecular weight than polymerized glutaraldehyde. *p*-Benzoquinone (PBQ), which can substitute for GA in two-step reactions, has an advantage in that it can also react with the antibody carbohydrate or sulfhydryl groups (43). Similarly, *N,N'-o*-phenylenedimaleimide (OPDM) can be used to cross-link via existing or introduced sulfhydryl groups, but under milder conditions than using GA or PBQ. Despite its advantages over GA and PBQ, it is not widely used for enzyme–antibody conjugation since some enzymes such as AP and β-glucosidase are inactivated.

Historically, the most popular coupling reaction has been via periodate oxidation of carbohydrate. The product of this reaction is unstable in long-term storage and the ratio of coupled enzyme to antibody cannot be controlled. A two-step modification of this procedure using reducing conditions gives greater control and stabilization (44) and yields useful conjugates at low cost. This method can be applied to any glycosylated enzyme and all mammalian cell expressed antibodies. Horseradish peroxidase is used most often, but glucose oxidase (45) has also yielded satisfactory conjugates.

The bifunctional ester bissuccinic acid *N*-hydroxysuccinimic ester (BSNHS) is used for conjugation of microperoxidase to antibodies. It gives high antibody activity because 8-microperoxidase has only one reactive amino group.

Coupling methods utilizing heterobifunctional reagents offer better conjugation because they avoid undesirable cross-linking or aggregation. A wide variety of heterobifunctional reagents are used, mostly based on *N*-succinimidyl esters that add aliphatic or aromatic groups containing thiol (46) or maleimide groups

(47). The activated ester couples via amino groups to provide an active group without introducing charged or hydrophobic residues. Combinations of maleimide substituted proteins and either natural or introduced thiol groups on the other protein give rise to controlled irreversible coupling under mild conditions. They function effectively to couple most of the common enzymes to antibodies (47).

In many circumstances the size of the conjugated antibody is critical. Because the Fc portion of the antibody is not required or is a disadvantage (increasing nonspecific signals by adsorption or cross-reactivity with heterophillic antibodies, rheumatoid factors, receptors, etc.), the use of antibody Fab fragments is advocated. This has the additional advantage that the Fab fragments express free thiol groups (derived from the hinge region) by which enzymes may be specifically attached using bifunctional linkage reagents employing maleimide. Since the hinge and the thiol groups are located distal to the antigen binding site, such conjugates have high specificity and activity, allowing improved sensitivities. This approach has not been widely applied because it is difficult to generate pure Fab or $F(ab')_2$ fragments from many antibody isotypes using proteolytic enzymes.

Since antibodies can recognize a number of distinct or overlapping epitopes on a protein surface, it is possible to produce antibodies against enzymes that do not interfere with enzyme activity upon binding. It is, therefore, possible to generate enzyme-antienzyme complexes that can be linked to bound primary antibody by an anti-Ig antibody reagent. This system has an advantage because it amplifies a signal, is not prone to inactive enzyme and antibody probes, and has minimum background signal. The peroxidase-antiperoxidase (PAP) (27) and alkaline phosphatase-anti-alkaline phosphotase (APAAP) systems are commonly used, particularly in immunohistochemistry assays (48), although some are used in enzyme immunoassays. Such complexes are easily solubilized and offer larger signals than those obtained by chemical coupling. They can also be added directly to the primary reagent, thereby reducing overall assay times.

Linkage of enzyme and antibody by auxillary molecules provides a potentially universal and variable number of systems for use in bioassays. The most common linkage system involves the recognition of biotin by avidin, or more commonly streptavidin (due to its higher affinity for biotin), although the use of staphylococcal protein A can be used for some antibody isotypes (11). An alternative system, but one that is not often used, involves liposomes loaded with enzyme that has antibody molecules on its surface (49).

5. DETECTION SYSTEMS

The majority of enzyme labels are detected using either colorimetric or fluorimetric methods. Instrumentation is available in a variety of formats to suit assays carried out in tubes, and on microtiter plates, dipsticks, and membranes. The principles of these detection methods are widely understood and their discus-

sion here will be restricted to the practical and theoretical limitations of the techniques. Chemiluminescence and bioluminescence reactions are becoming more common and therefore these detection methods will be discussed in more detail.

5.1. Colorimetric Detection of Enzyme Labels

Colorimetric detection relies on the enzymatic production or consumption of a molecule with a distinctive absorption spectrum. Measurement of reaction rate or extent is made via absorption measurements at or near the maximal absorption wavelength. The technique has several fundamental limitations that restrict its use in highly sensitive assays. However, it is undeniably the simplest method of assessing an assay endpoint, and suitable instruments are available in most laboratories.

Achieving maximal sensitivity in an enzyme assay requires that the absorption spectra of substrate and product do not overlap significantly. The difference in absorption coefficients at the chosen wavelength should be as high as possible so that a large absorbance change occurs on substrate conversion. Ideal substrates should absorb well into the visible portion of the spectrum to avoid interference by other molecules. Most practical immunoassay readers are limited to about 2.0 A full scale and cannot resolve absorbance changes less than 0.01 A. Light scattering by immunoassay solid phases or damaged cuvettes adversely effects the accuracy and precision of absorbance measurements and hence limits the sensitivity. This limitation can be negated if measurements are made at a second wavelength which is unaffected by the chromophore, but this facility is not available on all instruments.

5.2. Fluorescence Detection of Enzyme Labels

Detecting enzyme activity by fluorescence measurements relies on the production or consumption of fluorescent substrates or products. Fluorescence emission is directly related to concentration of the emitting substance; therefore, the method is mainly limited by the signal-to-noise ratio of the detector. With an efficient detector it is possible to determine compounds over a 5-order magnitude concentration range. Fluorescence must be driven using an excitation light source, usually incident at 90° to the detected fluorescence. Both excitation and emission wavelengths are selected using either filters or monochromators. In both cases some stray light can be scattered from excitation source to detector. The scattering observed depends on how close the excitation and emission wavelengths are to one another.

Probably the most fundamental limitation to the sensitivity of fluorescent detection is the problem of contaminating fluorophores. Bilirubin and many drugs have fluorescent metabolites. This interference is less important for determining labeled enzyme than for direct fluoroimmunoassay because of the amplification provided by the enzyme.

5.3. Chemiluminescent and Bioluminescent Detection of Enzyme Labels

Chemiluminescence is light emission that arises during the course of a chemical reaction, whereas bioluminescence is a special type of chemiluminescence found in biological systems in which a catalytic protein increases the efficiency of the chemiluminescent reaction. The principal advantages of employing such reactions to monitor immunoassays are that they are extremely sensitive and rapid. These reactions provide a very sensitive detection system because no external light source is required, as would be the case for fluorescence or colorimetric measurements. All the light reaching a detector originates from the chemical reaction. The instrumental background signal is essentially zero and hence the contribution of a single chemiluminescent reaction event can be detected as a single photon.

Luminescent endpoints are available for all of the commonly used enzyme labels, including horseradish peroxidase, alkaline phosphatase, glucose-6-phosphate dehydrogenase, glucose oxidase, and β-galactosidase. Detection limits in the subattomolar range have been attained (34). Chemiluminescent assays for novel enzyme labels such as xanthine oxidase have also been developed (50).

Alkaline phosphatase can be determined using the *o*-phosphate derivative of firefly luciferin as a substrate. Alkaline phosphatase cleaves the phosphate group to produce D-luciferin, which, unlike the *o*-phosphate derivative, reacts with firefly luciferase and ATP to produce light (51). In the chemiluminescent assay, an adamantyl 1,2-dioxetane aryl phosphate is the substrate. Alkaline phosphatase cleaves the phosphate group, producing an unstable intermediate, which decomposes with the emission of light. The reaction has been incorporated in a range of immunoassays (52, 53). Detection limits for the enzyme label using either substrate are in the femtomole–attomole range.

Horseradish peroxidase (HRP) is widely used as a label in immunoassays. The enzyme is rapidly and sensitively assayed by its ability to catalyze the chemiluminescent oxidation of a range of substrates, such as cyclic diacyl hydrazides, phenol derivatives, and certain components of bioluminescent systems. Peroxidase will catalyze light emission from luminol in the presence of hydrogen peroxide at pH 8.5. Under these conditions addition of any one of a series of substituted phenols, naphthols, and amines to the reaction mixture increases light emission over two orders of magnitude (54–56). The degree of enhancement and the kinetics of light emission are dependent on the individual enhancer, its concentration, the reaction conditions employed, and the purity of the diacyl hydrazide preparations (57). Background emission in the absence of HRP is also reduced by some compounds. These effects combine to produce a major improvement in the signal to background ratio and make the timing of reagent addition less critical.

Multiple reactions can be initiated manually prior to presentation to the detector. The endpoint may be applied to immunoassays using different solid supports and also adapted to Western blot analysis (58), Southern blot analysis,

and DNA probe assays (59–61). Enhanced chemiluminescence is also successfully used to assay for hepatitis B viral DNA in serum samples using solution hybridization and bead capture (62).

Chemiluminescent assays are conventionally monitored using photomultiplier-based instruments. However, portable instruments are becoming available that use as photodetectors silicon photodiodes, charge-coupled devices, and instant photographic or x-ray film (58, 63–68). Spacially resolved, quantitative light measurements are particularly advantageous for assays based on membranes or microtiter plates.

6. APPLICATION OF ENZYME-LABELED ANTIBODIES IN IMMUNOASSAYS

6.1. Laboratory-Based Systems and Applications

Enzyme-labeled antibodies are employed to measure microbial-, plant-, insect-, avian- and mammalian-derived biological targets (nucleic acids, proteins, lipids, etc.) and those derived from chemical syntheses. Immunoassay formats utilizing enzyme–antibody conjugates can be divided into those involving limited reagent (i.e., competitive) or excess reagent (i.e., noncompetitive) and involving separation (heterogeneous) or nonseparation (homogeneous) of reactants from product. The most widely adopted heterogeneous assay format is the ELISA (enzyme-linked immunosorbent assay). Within this category the format is essentially equivalent, although it may be based on antigen capture, competition, antibody capture, or buildup of multiple layers, or involve dual labels.

The development of two-site "sandwich" assays based on the use of antibodies specific for spatially discrete sites on the target analyte has increased the variety, sensitivity, and specificty of such assays. It has also maximized signal-to-noise ratios and allowed the use of high reagent concentrations to minimize the importance of sensitivity limits imposed by antibody affinity, a problem often encountered in developing competitive assays. This format is particularly suited to the sensitive detection of an analyte in a sample and has the advantage that relatively small sample volumes can be used. The range of analytes detectable in ELISA formats is large, and the sensitivity is generally higher than would be possible in a single-antibody method. The presence of excess antibody means that the analyte binding equilibrium is biased in favor of the bound state, allowing binding of essentially all of the analyte even at concentrations significantly lower than the antibody binding constant. The excess capacity also allows interference-free assays in the presence of relatively high concentrations of similar molecules, providing the interferant is bound by only one of the antibodies. The need for an immobilized capture layer means that generally sensitivities are limited by background signals due to inappropriate binding of label to the solid-phase and sample-induced interference (such as binding of heterophilic antibody or rheumatoid factor to both antibody-coated solid phase and labeled antibodies). The signal-to-noise ratio is increased along with specificity

by the use of two cooperative monoclonal antibodies immobilized to the solid phase, each recognizing a distinct epitope; A third enzyme-labeled antibody recognizing a distinct epitope is used to reveal the bound analyte. The use of two distinct capture antibodies gives the assay a broader detection range by increasing the apparent affinity constants of the antibodies, allows shorter reaction times, and can recreate desirable cross-reactivity to emulate the characteristics of polyclonal antisera. Enzyme-antibody conjugates are also used in formats for detecting nucleic acid hybrids. The specificity of the antibody for hetero- (RNA:DNA) and homo-duplexes (DNA:DNA) is utilized as a universal indicator for complementary hybridization of specific probes to nucleic acid sequences.

There are relatively few homogeneous assay systems currently utilized in the laboratory, because development costs for sensitive assays are often prohibitive and the increasing extralaboratory test market (where sensitivity is generally unimportant) has been the main inducement for their development. This form of assay utilizes the modulation (increase or decrease) of the enzyme activity by the binding of the antigen as the means of avoiding a separation step. They are simple and rapid but not sensitive: They are used to measure drugs of abuse and therapy in blood and urine. Using these assays is hampered by the fact that it is difficult to achieve the optimal 100% modulation of the enzyme-mediated signal.

CEDIA is a homogeneous assay based on a novel principle. The β-galactosidase gene from *E. coli* is cloned and genetically engineered into two inactive fragments, the enzyme donor (ED) and enzyme acceptor (EA). These normally spontaneously combine to recreate active enzyme. In the assay the ED fragments are conjugated to the drug of interest and bind to the antibody, preventing enzyme reactivation. Drug molecules added with the sample result in displacement of the labeled ED and hence increased enzyme activity (69). This assay has been applied to a number of haptens and is easily automated, because it only requires pipetting of sample and two reagents followed by absorbance reading in a kinetic mode for 210 s. It represents the first example of what will probably be a large number of genetically engineered detection systems.

Using enzyme-antibody labels to detect immobilized analytes as imprints from gel-separated samples has expanded in the last decade. Blotting technologies, Western (protein), Southern (DNA), Northern (RNA), or vacuum (DNA, RNA, or protein), have to varying degrees used enzyme-antibody conjugates. Most blot immunoprobing methodologies such as ELISA follow the same format and generally utilize the same enzyme labels, but with a variety of endpoints, including colorimetry, bioluminescence, chemiluminescence, and fluorescence. Enzyme-labeled antibodies have an advantage over radioactive labels because of the speed of signal generation. What they had previously lacked in sensitivity over radiolabels has been diminished by the development of signals of greater intensity per bound enzyme label (e.g., chemiluminescent) and signal amplification systems (70). Visualizing the blot directly instead of trying to match up the autoradiograph pattern with the original stained blot provides an additional advantage.

6.2. Cell-Based Assays and Applications

Antibodies, particularly since the availability of monoclonal antibodies, are widely applied in the assay of cells. In very broad terms, cell-based assays may be regarded as assays that measure an analyte in a sample containing cells. Using this definition makes it possible to divide them into three categories: (1) those in which the analyte is expressed on the cell surface, (2) those where the analyte is expressed intracellularly, and (3) those where the analyte is secreted by the cell during the period of the assay. Each type of assay imposes constraints on the assay format and the type of antibody probe that can be used. For example, flow cytometry based assays are best suited to fluorescently labeled antibodies. Major disdavantages of antibody–enzyme conjugates in cell assays are the presence of endogenous enzymes and the relatively large size of the conjugate. We shall now consider examples of each of these three types of cell-based assay and discuss the relative merits and limitations of enzyme-labeled antibodies.

The detection of cell surface analytes has many fundamental and clinical applications. The cell surface is a very mobile phase composed of lipids with different types of integral protein and carbohydrate moieties that may be cell specific or co-expressed by a number of cell types. Such molecules range from the histocompatibility antigens to numerous types of cellular receptors. Furthermore, these molecules may be expressed at different times during development or cell differentiation, and in different numbers in response to physiological or pathological stimuli. These facts are important to understanding normal physiological and pathological processes and regulatory mechanisms. Cell surface antigens therefore represent important markers in terms of diagnosis, therapy monitoring and prognosis, and prevention of certain pathological phenomena (i.e., identifying susceptibility to deleterious reactions such as incompatability reactions) allowing detection and enumeration of different cell types or surface-expressed antigens (e.g., viral proteins). However, the utility of enzyme–antibody probes in such circumstances has been limited to a few research systems. Only in rare cases is it important to detect the presence of an antigen-bearing cell among a population of negative responders. Under normal conditions the result must be expressed as a proportion of total cells and hence the detected signal must remain closely localized with the cell of origin. Only physically linked signals from fluorophore or short lifetime chemiluminescent labels can achieve this because colored products of enzyme label-catalyzed reactions are long-lived and are free to diffuse away from their source. Many enzymes used as antibody labels are also expressed by different cell types, thus raising the background signal level and lowering the sensitivity of the assay. The major application of enzyme-labeled antibodies for surface antigen expression therefore remains in formats similar to those for soluble analyte detection, that is, cell ELISA systems. Alternatively, they have limited application in immunohistochemistry formats for immunolocalization of cell surface-expressed antigens. Such applications are in the microscopical screening of cells from smears or cytospins where identification of cell morphology and antigen expression is required in samples containing relatively few cells, for example, cervical smears. In these circumstances,

the signal sensitivity of the assay is less important than the ability to reliably detect aberrant cells with high sample throughputs and minimal instrumentation is important.

Enzyme labeled antibodies currently have a significant application in the immunolocalization of intracellular antigens or analytes. There is a distinction between direct detection of an antigen and the indirect detection of an analyte. In the former situation, the enzyme-antibody probe is most useful for localizing structural features that are characteristic of tissue sections. In the latter, the probe is generally applied to detect a nonstructural component by signal amplification, such as nucleic acid. We refer here to the detection of mRNA or DNA sequences by in situ hybridization, where target sequences are specifically recognized by a biotinylated oligonucleotide probe and the antibody-enzyme probe is used to construct the layers of the signal amplification system, for example, PAP/APAAP. In either case, the utility of enzyme-antibody conjugates is severely hampered by the conjugate size, which may limit the permeability of the complex into the tissue section. This limitation is reduced somewhat by the application of microperoxidase conjugates (71), but is still limited by the endogenous expression of similar enzyme activities in the sample tissue.

Finally, enzyme-antibody labels are used to detect soluble analytes secreted by cells. In these circumstances the format is similar for the detection of the analyte in a cell-free sample (i.e., in an ELISA system), but it requires some modification to allow individual sources of the analyte to be identified. Examples of such an assay are those reported by Skidmore et al. (72) and Sedwick and Holt (73) for enumeration of interferon-gamma and tumor necrosis factor-secreting cells and antibody-secreting B cells, respectively, in samples of peripheral blood or tissue extracts. In these assays, microplate formats akin to ELISA were used with the difference that microscopic visualization of the insoluble colorimetric signal served as the endpoint.

7. NEW PERSPECTIVES

Two mutual areas of development, in reagent or assay design, can be predicted to have a considerable effect on the utilization of enzyme-labeled antibodies in bioanalytical systems: (1) genetic engineering of antibodies and enzymes, and (2) simplified assay methodologies and instrumentation. However, genetic engineering has potentially the greatest role in influencing overall developments, while advances in assay and instrument design have the greatest effect on the ultimate range of analytes and situations to which such assays may be applied.

Developments in enzymes and antibodies have been paramountly affected by the diversity of the genetic repertoire of nature and an increase in our ability to understand, manipulate, and release this by the techniques of recombinant genetics. The traditional interest in enzymes stemming from their diversity, ubiquity, breadth of applications, and biological importance made them an obvious initial target for the early pioneers of in vitro genetic recombination.

Initial efforts have concentrated on the functional aspects of enzymes, that is, their active sites. While nature has provided a wealth of enzymes with varying substrate activities, there exists a need for additional sources and types of enzymes with higher turnover rates and substrate specificities. This has resulted from the fact that many commercially valuable enzymes are, in economic terms at least, less efficient than desired, often needing co-factors as catalysts. Based on sequence:structure:function data, site-directed mutagenesis has generated new variants with higher substrate binding affinities.

Another avenue of investigation has been to examine the effect of reducing enzyme size almost down to the minimal active site. Deletional mutagenesis of cloned enzymes has revealed that much of the structural sequence of enzymes can be lost, or replaced, while still retaining catalytic activity. It is therefore now possible to create fusion proteins between different enzymes such that a bi-specific reagent is created, that is, one having two dissimmilar active sites, akin to the methods for creating bi-specific antibodies (see Fig. 1). Following on from this one can create all sorts of molecules with desirable attributes in terms of size, shape, linkage sites, specificities, affinities, and so on. In the context of this chapter we shall consider these specifically in the context of antibody labels and their role in determining the future of bioassay design.

The constitution of immunoglobulin genes and their recombination in individual B lymphocytes to generate any desirable specificity has been the focus of much investigation. Although all the facets of its operation are not yet fully deciphered, more recently this knowledge has been utilized by molecular biologists for in vitro selection of antibody functions. The genetic basis of antibody diversity of function can be briefly described as follows.

Each antibody molecule is encoded by seven gene segments spatially discrete within the genome (VL, JL, CL, VH, JH, DH, CH) and each of these are part of large families of genes. The numbers of genes within a family may vary between species but the overall composition and selection mechanisms appear to be equivalent. The recombination between members of these gene families is the first level at which the diversity of functions and antigen specificities is generated. The selection of D (or diversity) gene segments increases antibody diversity and this is enhanced by the fact that in recombining, the junction between gene V, J, and D segment is imprecise, leading to loss of amino acids or frame shifts, causing amino acid substitutions. As yet undefined mechanisms also increase antigen specificity diversity and affinity by (semi-)random mutation within the variable region genes following rearrangement.

Thus, the domain structure of antibodies with separating peptide sequences correlates with immunoglobulin gene organization and assembly, potentially allowing shuffling and splicing of alternative gene segments. This has already been achieved and demonstrated by several research groups (for a review, see Winter and Milstein (2)) and includes the generation of chimearic antibodies (see Fig. 2) with variable region specificities from one animal species grafted into constant and framework sequences derived from another animal species. Other recombinant antibodies have been constructed that have the positions of in-

dividual domains exchanged, for example, human IgE molecules with Cε2 and Cε3 interchanged in order to map receptor binding sites. Structural features such as the hinge region have also be modified by genetic engineering, and more recently it has been demonstrated that a single cloned variable domain may be expressed with full retention of antigen binding and affinity (74). Furthermore, in vitro construction and expression of single domains in lambda phage libraries (75), combined with hypermutation studies have enabled a much wider range of specificities to be achieved from the immunoglobulin repertoire than it has been possible to generate from immunization of animals. An additional potential advantage accruing from such studies is that the requirement for animals may ultimately be obviated. The cost and standardization of antibody reagents should also be greatly facilitated. The fact that transgenic plants have been generated that express intact, functionally active antibody protein (13) suggests that in the future antibodies could be harvested as a by-product of existing agricultural practice!

The impact of such molecular biological manipulations has as yet been limited in terms of bioassay design. However this will not remain so for long. Based on the present demonstrations and theoretical conjecture, many novel antibody reagents may be anticipated that are relevant to the future of antibody–enzyme conjugates and their applications.

Antibodies and enzymes are not dissimilar in that they are both binding agents. That is to say, the most basic function of an antibody is to recognize an antigen through complimentarity of the antibody's antigen binding structure with the surface topography of the antigen. A prerequisite of any enzyme is that it must also bind substrate in an active site/cleft; indeed, the nature of enzyme and antibody active sites is often very similar. This factor, plus the inherent variability of antibody specificities, has prompted many groups to investigate the possibility of raising catalytic antibodies, that is, antibodies with antigen binding sites that are able to function as substrate binding sites with attendant enzymic activity (76). Since nature has evolved immunoglobulin genetics to develop a rapid and adaptable response to any change by generating the desired specificity, this can potentially be exploited to generate a wide range of enzyme functions rapidly without the considerable effort required to perform this by traditional mutagenesis methods. This goal has been proposed predominantly for generating enzyme activities to meet the demands of biotechnology processes, for example, fermentation and manufacturing pharmaceuticals. Although such useful antibodies have as yet largely eluded researchers, it would be prudent to consider the implication that such molecules could have for bioassays. For example, the concept is not far removed from the fact that catalytic antibodies could be produced that also retain antigen binding specificity. In such cases, antigen may activate or inactivate the enzymic activity leading to quantifiable changes proportional to the analyte concentration. The fact that small but detectable changes in Fab conformation have been described between the antigen bound and unoccupied states suggests that such molecules can be created and exploited.

In the same vein, the fact that genetic engineering can generate minimal sized enzymes and antibodies (i.e., single variable region domains or minimal recognition units (mru) (2)) can ultimately be used to create chimearic molecules that have discrete antigen and substrate binding sites. Even if they are considered as whole molecules, fusion proteins whereby an antibody domain is substituted for by an enzyme moiety would have considerable impact in that second reagents may be unnecessary. Sensitivities of assays may also be increased by constructing antibodies with enzyme multimers replacing each of the constant domains, yielding potentially six labels per antibody compared to the present average of three achieved by chemical labeling. If you add the possibility that enzymes may be generated that have more than one active site, then the equation is biased toward sensitivity; if two types of active site are created, then integral dual labeling or linked reactions are feasible. Bioassays could then be much more simple, sensitive, inexpensive, and flexible. Since such chimaeric proteins would most likely be expressed in prokaryotic systems on a large scale, they would also become more standardized and cheaper.

Expanding our conjecture in a slightly different direction, one can consider the effect of introducing other types of enzyme activities into the integral structure of antibodies. Inclusion of protease, glycosidase, or lipase functionalities into antibodies could have profound effects for multianalyte bioassays, in fundamental and applied research, such as in vivo diagnostics and therapeutics. Humanization of murine monoclonal antibodies has already aided research into physiological and pathophysiological functionalities, and humanized antibodies have been successfully used in vivo for therapy. Effort toward the "magic bullet" for in vivo destruction of diffuse and metastatic tumor cells is also directed toward the development of antibody–enzyme fragment conjugates such that enzyme activation requires antigen interaction or use of a secondary reagent bearing the essential co-factor for activation. Such conjugates may also enhance the ability to dictate specifically the compartmentalization of the reagents (i.e., to prevent nonspecific uptake by normal cells and tissues) and, by choosing the appropriate enzyme, prevent undesired activation of ubiquitous sources of the enzyme. However, much fundamental experimentation is required before the theoretical becomes the practical. Considerations such as the inherrent instability of recombinant prokaryotic expressed proteins (e.g., *E. coli* expressed antibody Fv, which is unstable at physiological temperatures) must be overcome to allow realistic and expansive exploitation.

SUMMARY

Antibody–enzyme conjugates are widely utilized in all spheres of specific analyte detection and measurement, and several trends are evident that will sustain, or even extend, this in the coming years. Of principal importance are the trends toward the development of simplified formats for the rapid and sensitive quan-

titation of a wide range of analytes without expensive or cumbersome instrumentation, and the exploitation of different types of enzyme and antibody molecules. Advances in hybridoma and recombinant genetics are enabling the practical manipulation of the theoretical repertoire of these reagents, facilitating their availability for a myriad of applications.

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