Kirk-Othmer Encyclopedia of Chemical Technology.

BIOPOLYMERS, ANALYTICAL TECHNIQUES

Analytical techniques that utilize biopolymers, ie, natural macromolecules such as proteins, nucleic acids, and polysaccharides that compose living substances, represent a rapidly expanding field. The number of applications is large and thus uses herein are limited to chiral chromatography, immunology, and biosensors.

1. Biopolymers in Chiral Chromatography

Biopolymers have had a tremendous impact on the separation of nonsuperimposable, mirror-image isomers known as enantiomers. Enantiomers have identical physical and chemical properties in an achiral environment except that they rotate the plane of polarized light in opposite directions. Thus separation of enantiomers by chromatographic techniques presents special problems, whereas diastereomers have different chemical and physical properties and may be separated by more conventional methods (see Chromatography). Direct chiral resolution by liquid chromatography (lc) involves diastereomeric interactions between the chiral solute and the chiral stationary phase. Because biopolymers are chiral molecules and can form diastereomeric interactions with chiral solutes, they are ideal for use as chiral stationary phases. This property has led to a rapid growth of chromatographic stationary phases utilizing biopolymers to separate chiral molecules.

1.1. Cyclodextrin Chromatographic Phases

1.1.1. Properties and Structure

Cyclodextrins (CDs) are natural macrocyclic polymers of glucose that contain from 6 to 12 D-(+)-glucopyranose units bonded through α-(1,4)-linkages. They are chiral, torodial-shaped molecules with all the glucose units in a C-1 (D) chair conformation (1, 2). The structure of β-cyclodextrin is shown in Figure 1. The mouth of the molecule, facing outward, has the larger circumference of the openings, and contains the secondary hydroxyl groups on C-2 and C-3 of the glucose molecule. The primary hydroxyl groups, those attached to C-6 of the glucose unit, are on the opposite end of the cyclodextrin, which forms the smaller opening. The cyclodextrin molecule is therefore shaped like a truncated cone. The primary hydroxyl groups on the truncated end can rotate to block the cavity partially, whereas the secondary hydroxyl groups at the mouth are held relatively rigid. The interior of the cavity consists of two rings of C–H groups with a ring of glucosidic oxygens in between. This makes the interior relatively hydrophobic in comparison to polar solvents such as water. The mouth of the cyclodextrin cavity, however, is hydrophilic. A Greek letter is used to denote the number of glucose units in the cyclodextrin molecule. For example, α for six, β for seven, γ for eight, and so on.

Some physical properties of the four most common cyclodextrins are listed in Table 1 (3). Other important properties are (*1*) cyclodextrins are nonreducing; (*2*) glucose is the only product of acid hydrolysis; (*3*) molecular weights are always integral numbers of 162.1, the value for glucose; (*4*) cyclodextrins are nontoxic; and (*5*) they do not appreciably absorb ultraviolet (uv) or visible light.

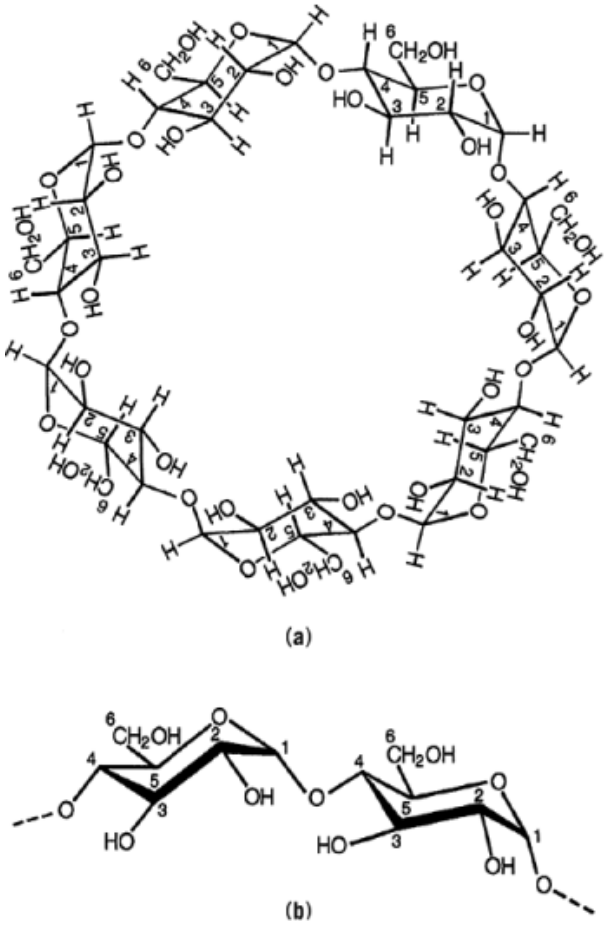

Fig. 1. Structural diagram of (**a**) β-cyclodextrin and (**b**) two of the glucopyranose units illustrating details of the α-(1,4) glycosidic linkage, C-1 (D) chair conformation, and the numbering system employed to describe the ring system.

Table 1. Physical Properties of Cyclodextrins

Cyclodextrin	CAS Registry Number	Molecular formula	Number of glucose units	Cavity diameter, nm[a]		Aqueous solubility, *M*
				External	Internal	
α-CD	[10016-20-3]	$C_{36}H_{60}O_{30}$	6	1.37	0.57	0.114
β-CD	[7585-39-9]	$C_{42}H_{70}O_{35}$	7	1.53	0.78	0.016
γ-CD	[17465-86-0]	$C_{48}H_{80}O_{40}$	8	1.69	0.95	0.179
δ-CD	[85220-53-7]	$C_{54}H_{90}O_{45}$	9			v sol

[a] Depth of cavity is 0.78 nm for each cyclodextrin.

The enzyme responsible for producing cyclodextrins from starch, cyclodextrin transglycosylase (CTG), does not cleave a specific number of glucose units from starch. Homologues from 6 to 12 glucose units may be obtained as mixtures having small amounts of branched cyclic molecules and branched open-chain dextrins.

A five-membered or smaller dextrin ring has never been obtained. This is most likely attributable to the considerable strain present in small rings.

Several procedures are used to control the ratios of cyclodextrins produced. One is addition of a substance to the reaction mixture that can greatly affect the formation of one specific cyclodextrin over another. For example, in the presence of 1-decanol and 1-nonanol, α-cyclodextrin is produced almost exclusively whereas hexane or toluene promote the production of β-cyclodextrin. Conversely both cyclodextrins are produced simultaneously in the presence of 1-heptanol (2, 4).

1.1.2. Immobilization

The ability of cyclodextrins to form inclusion complexes selectively with a wide variety of guest molecules or ions is well known (1, 2) (see Inclusion compounds). Cyclodextrins immobilized on appropriate supports are used in high performance liquid chromatography (hplc) to separate optical isomers. Immobilization of cyclodextrin on a solid support offers several advantages over use as a mobile-phase modifier. For example, as a mobile-phase additive, β-cyclodextrin has a relatively low solubility. The cost of γ- or α-cyclodextrin is high. Furthermore, when employed in thin-layer chromatography (tlc) and hplc, cyclodextrin mobile phases usually produce relatively poor efficiencies.

Cyclodextrin stationary phases utilize cyclodextrins bound to a solid support in such a way that the cyclodextrin is free to interact with solutes in solution. These bonded phases consist of cyclodextrin molecules linked to silica gel by specific nonhydrolytic silane linkages (5, 6). This stable cyclodextrin bonded phase is sold commercially under the trade name Cyclobond (Advanced Separation Technologies, Whippany, New Jersey). The vast majority of all reported hplc separations on CD-bonded phases utilize this media which was also the first chiral stationary phase (csp) developed for use in the reversed-phase mode.

1.1.3. Applications

The first widely applicable lc separation of enantiomeric metallocene compounds was demonstrated on β-CD bonded-phase columns. Thirteen enantiomeric derivatives of ferrocene, ruthenocene, and osmocene were resolved (7). Retention data for several of these compounds are listed in Table 2, and Figure 2**a** shows the lc separation of three metallocene enantiomeric pairs. β-Cyclodextrin bonded phases were used to resolve several racemic and diastereomeric 2,2-binaphthyldiyl crown ethers (9). These compounds do not contain a chiral carbon but still exist as enantiomers because of the staggered position of adjacent naphthyl rings, and a high degree of chiral recognition was attained for most of these compounds (9).

The β-CD column exhibits excellent selectivity for enantiomers of certain amino acid derivatives. Underivatized amino acids are apparently too small to bind tightly to the β-CD cavity and show no enantiomeric resolution. When a substituent such as a dansyl group is present on the amino acid, strong inclusion complexes with β-CD are formed and baseline separation is achieved (see Table 2) (10). Either the amino or the carboxylate group of the amino acid can be derivatized to obtain chiral recognition. Derivatization of both groups, however, tends to reduce chiral recognition. It is possible to detect as little as 0.2% of one enantiomer in a racemic mixture as shown in Figure 2**b**, thus providing an extremely sensitive test of optical purity (5).

Table 2 gives chromatographic data for different classes of enantiomeric drugs resolved by β-CD bonded phases (8). Drugs for which resolution factors (R_s) greater than 1.0 were obtained include mephenytoin, ketoprofen, chlorpheniramine, and the barbiturates mephobarbital and hexobarbital. Cyclodextrin-bonded phases provide a rapid and specific technique for the pharmacological evaluation of racemic drugs.

Many diastereomers, geometric isomers, and epimers can be successfully resolved using cyclodextrin phases (5, 10). For example, the four epimers of estriol [50-27-1], $C_{18}H_{24}O_3$, were separated using β-CD, and *cis*-benzo(*a*)pyrene and *trans*-benzo(*a*)pyrene were completely resolved on a γ-CD column. Diastereomeric drugs such as the cinchona alkaloids (qv) and antiestrogens have also been separated. Cyclodextrin columns are also of great utility in separating structural isomers such as the ortho-, meta-, and para- isomers of

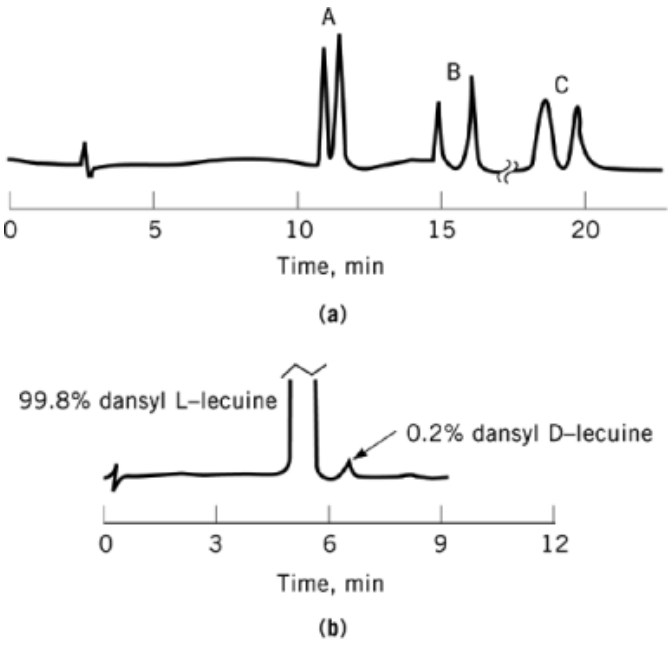

Fig. 2. Chromatogram showing (**a**) the lc separation of A, (±) (*S*)-(1-ferrocenyl-ethyl)thioethanol; B, (±) 1-ferrocenyl-1-methoxyethane; and C, () 1-ruthenocenylethanol, on a 25-cm β-cyclodextrin column (see Table 2), and (**b**) the potential use of a β-cyclodextrin column to determine optical purity when one of the enantiomers is present at very low concentration (5, 7).(Courtesy of Preston Publications.)

nitroaniline, xylene, cresols, nitrophenols, and substituted benzoic acids (11). Cyclodextrin bonded phases are used as nonconventional reversed phases for routine analyses as a result of the unusual selectivity of the cyclodextrin columns. Uses in routine analyses include the separation of a series of barbiturates, mycotoxins, polycyclic aromatic hydrocarbons, vitamins (qv) and selected dipeptides (12). Cyclodextrin stationary phases can be used in the normal-phase mode with hexane–isopropanol mobile phases. Solutes adsorb to the hydroxyl groups on the outside of the cyclodextrin, rather than forming inclusion complexes. Separations in the normal-phase mode tend to be analogous to those of diol columns.

1.1.4. Mechanism of Separation

There are several requirements for chiral recognition. (*1*) Formation of an inclusion complex between the solute and the cyclodextrin cavity is needed (4, 10). This has been demonstrated by performing a normal-phase separation, eg, using hexane–isopropanol mobile phase, on a β-CD column. The enantiomeric solute is then restricted to the outside surface of the cyclodextrin cavity because the hydrophobic solvent occupies the interior of the cyclodextrin. (*2*) The inclusion complex formed should provide a relatively "tight fit" between the hydrophobic species and the cyclodextrin cavity. This is evident by the fact that β-CD exhibits better enantioselectivity for molecules the size of biphenyl or naphthalene than it does for smaller molecules. Smaller compounds are not as rigidly held and appear to be able to move in such a manner that they experience the same average environment. (*3*) The chiral center, or a substituent attached to the chiral center, must be near to and interact with the mouth of the cyclodextrin cavity. When these three requirements are fulfilled the possibility of chiral recognition is favorable.

The unidirectional 2- and 3-hydroxyl groups located at the mouth of the cyclodextrin cavity appear to be of particular importance in chiral recognition. This is seen in Figure 3, which shows computer-generated

Table 2. Retention Data for Racemic Compounds Separated on a β-Cyclodextrin Stationary Phase[a]

Compound	CAS Registry Number	Molecular formula	k′[b]	α[c]	R_s[d]
		Complexes			
1-ferrocenyl-1-methoxy-ethane		$C_{13}H_{17}FeO$	3.8	1.12	1.58
(*S*)-(1-ferrocenylethyl)-thioethanol		$C_{14}H_{17}FeSO$	3.0	1.23	2.13
1-ruthenocenylethanol		$C_{12}H_{14}RuO$	4.6	1.11	1.56
alanine β-naphthylamide	[74144-49-3]	$C_{13}H_{14}N_2O{\cdot}HCl$	5.1	1.20	2.0
dansyl-leucine	[102783-70-0]	$C_{18}H_{24}N_2O_4S{\cdot}C_6H_{13}N$	3.0	1.40	2.4
dansylphenylalanine	[42808-06-0]	$C_{21}H_{22}N_2O_4S{\cdot}C_6H_{13}N$	3.1	1.23	1.1
		β-Adrenergic blocker			
propranolol hydro-chloride	[3506-09-0]	$C_{16}H_{21}NO_2{\cdot}HCl$	2.78	1.04	1.40
		Antihistamine			
chlorpheniramine	[132-22-19]	$C_{16}H_{19}ClN_2$	5.86	1.07	1.51
		Sedative–anticonvulsants			
mephenytoin	[50-12-4]	$C_{12}H_{14}N_2O_3$	0.48	1.33	1.83
mephobarbital	[115-38-8]	$C_{13}H_{14}N_2O_3$	14.80	1.14	1.60
		Nonsteroidal antiinflammatory			
ketoprofen	[22071-15-4]	$C_{16}H_{14}O_3$	7.67	1.06	1.24

[a] Refs. (4, 5), and 8.
[b] k′ is the capacity factor for the first eluting isomer.
[c] α is the selectivity factor and is the ratio of the capacity factor of the last eluting isomer to the first eluting isomer.
[d] R_s is the resolution factor. $R_s = 2$ (distance between peaks)/(sum of the bandwidths of the two peaks).

projections of the lowest free-energy inclusion complexes of (*R*)- and (*S*)-propranolol with β-CD (8). The (*R*)- and (*S*)-propranolol are placed identically inside the cyclodextrin cavity and the hydroxyl groups attached to the chiral carbon for the enantiomers are placed in the same position for ideal hydrogen bonding to the 3-hydroxyl group of the cyclodextrin. Important differences exist between the complexes with respect to the secondary amine group. In the (*R*) complex the respective bond distances between the nitrogen and the cyclodextrin 2- and 3-hydroxyl groups are 0.33 and 0.28 nm, respectively. This allows for two reasonable hydrogen bond interactions. The same amine group in the (*S*)-propranolol complex is positioned less favorably for hydrogen bonding: closest bond distances are 0.38 and 0.45 nm, respectively. These models suggest that (*R*)-propranolol can preferentially interact with β-cyclodextrin in a way that the (*S*)-isomer cannot, resulting in chiral recognition by the cyclodextrin molecule. These findings agree with the three-point attachment concept introduced in the 1950s (13).

1.2. α_1-Acid Glycoprotein Chromatographic Phases

1.2.1. Properties and Structure

α_1-Acid glycoprotein (α_1-AGP) has a molecular mass of about 41,000 and consists of a peptide chain having 181 amino acid residues and five carbohydrate units (14, 15). Two cystine disulfide cross-linkages connect residues 5 and 147 and residues 72 and 164. The carbohydrate units comprise 45% of the molecule and contain sialic acid, hexosamine, and neutral hexoses. In phosphate buffer the isoelectric point of the protein is 2.7. AGP is a very stable protein and tolerates organic solvents as well as high temperatures. AGP columns may be used over a wide pH range without being denatured. Denaturation, as measured by changes in the optical rotation of the molecule, may be caused by boiling in distilled water or adding 10 *M* LiBr, 10 *M* urea, or 5 *M* HCl (14).

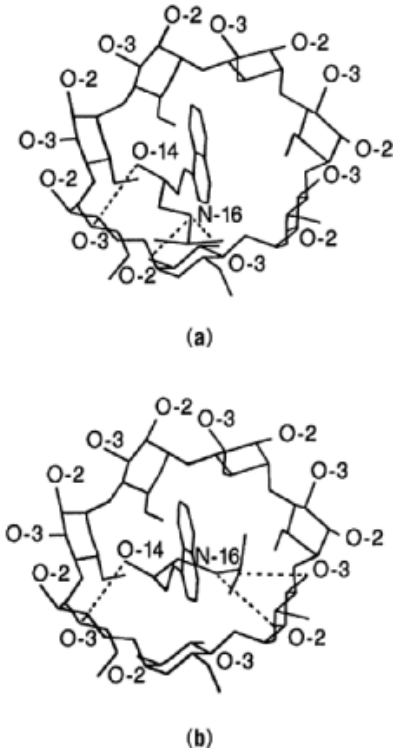

Fig. 3. Computer projections of β-cyclodextrin inclusion complexes of (**a**) (R)-propranolol and (**b**) (S)-propranolol from x-ray crystallographic data. Dotted lines represent potential hydrogen bonds (see text). The configurations shown represent the optimal orientation of each isomer on the basis of the highest degree of hydrogen bonding and complexation (8).

1.2.2. Immobilization

The solid-phase support used for bonding AGP is silica gel. The general preparation is based on bonding the protein by charge forces to diethylaminoethylsilica followed by a cross-linking procedure (16). A final reduction to secondary amines is performed using cyanoborohydride. The silica has a particle diameter of 10 μm, a large pore volume, and a large surface area. The commercially available EnantioPac AGP column (LKB Products, Bromma, Sweden) is manufactured using this procedure. A second generation AGP column, Chiral-AGP (ChromTech AB, Norsburg, Sweden), uses covalent linkage of the protein onto silica with cross-linking of adjacent protein molecules (17, 18). This latter packing contains 5 μm spherical silica particles having a smaller pore volume and a smaller surface area giving a stationary phase that is more mechanically stable and resistant to hydrolytic attack. Bonding capacity of the chiral phase and loading capacity of solute are directly dependent on the amount of protein that is bound. The amount of AGP bound on the silica depends on the bonding technique and on the accessible surface area of the silica.

1.2.3. Applications

Retention and selectivity of solutes on AGP is regulated by the concentration and properties of modifiers in the aqueous-based mobile phase. Retention and enantioselectivity usually decrease with increasing concentration of uncharged organic modifiers such as methanol, ethanol, 2-propanol, and acetonitrile in the mobile phase. However, in some cases, enantioselectivity can be improved by adding uncharged modifiers to the mobile phase (19). For example, addition of 2-propanol as an organic modifier gave improved chiral resolution for

Table 3. Baseline-Resolved Racemic Compounds Using Chiral-AGP

Compound	CAS Registry Number	Molecular formula	k′[a]	α[b]
alprenolol	[13655-52-2]	$C_{15}H_{23}NO_2$	17.9	1.19
atenolol	[2180-92-9]	$C_{14}H_{22}N_2O_3$	2.91	1.36
bupivacaine	[29122-68-7]	$C_{18}H_{28}N_2O$	6.66	1.29
cyamemazine	[3546-03-0]	$C_{19}H_{21}N_3S$	5.95	1.55
ephedrine	[90-81-3]	$C_{10}H_{15}NO$	3.86	1.34
ketamine	[6740-88-1]	$C_{13}H_{16}ClNO$	7.00	1.26
metoprolol	[37350-58-6]	$C_{15}H_{25}NO_3$	4.72	1.26
oxprenolol	[6452-71-7]	$C_{15}H_{23}NO_3$	16.1	1.29
pheniramine	[86-21-5]	$C_{16}H_{20}N_2$	11.3	1.33
pindolol	[13523-86-9]	$C_{14}H_{20}N_2O_2$	13.9	1.21
verapamil	[52-53-9]	$C_{27}H_{38}N_2O_4$	15.6	1.32
warfarin	[81-81-2]	$C_{19}H_{16}O_4$	8.18	1.39

[a] k′ is the capacity factor for the first eluting isomer.
[b] α is the selectivity factor See Table 2.

mephenytoin [50-12-4], $C_{12}H_{14}N_2O_2$, and methylphenobarbital. On the other hand, the enantioselectivity for the tertiary amines mepivacaine and bupivacaine is unaffected by additions of up to 8% of propanol to the mobile phase, despite the fact that the retention of these solutes decreases markedly under these conditions (18) (see Anesthetics; Hypnotics, sedatives, and anticonvulsants).

Column retention is generally increased if the modifier has a charge opposite to that of the solute; a modifier having the same charge as the solute generally decreases retention. For some compounds the addition of charged modifiers is essential to achieve chiral recognition. The fenthiazin derivative, propiomazine [362-29-8], $C_{20}H_{24}N_2OS$, was not resolved on an AGP column when phosphate buffer, pH 7.55, was used as the mobile phase. After addition of 1 m*M* of the tertiary amine *N,N*-dimethyloctylamine (DMOA) to the mobile phase, however, the enantiomers of propiomazine were baseline resolved (20) and longer retention times were obtained. Increase in retention is believed to be caused by competition between the solute and DMOA in binding to the AGP. The changes in selectivity are believed to result from reversible changes in the conformation of AGP brought about by changes in pH. Similar effects on selectivity and retention have also been observed by addition of tetrapropylammonium bromide or tetrabutylammonium bromide (15, 16, 21, 22).

Changes in the pH and temperature of the mobile phase can have a significant effect on both retention and selectivity. Changing the pH of the mobile phase has a profound effect on retention and selectivity for basic, acidic, and nonprotolytic compounds. For example, the separation factors for hexobarbital [56-29-1], $C_{12}H_{16}N_2O_3$ (weakly acidic) and metoprolol [37350-58-6], $C_{15}H_{25}NO_3$ (basic) (see Cardiovascular agents), increase with increasing pH, but the enantioselectivity for stronger acids such as 2-phenoxypropionic acid (pK_a = 4.6) generally decreases (21). Column temperature also strongly influences retention and enantioselectivity. It has been generally reported that retention, resolution, and separation factors decrease with increasing temperature, whereas efficiency increases.

AGP columns have wide application for the direct separation of enantiomers of many different classes of drugs, amines, acids, and nonprotolytic compounds (18, 23). Acidic drugs resolved include ibuprofen [15687-27-1], $C_{13}H_{18}O_2$, ketoprofen [22071-15-4], $C_{16}H_{14}O_3$, and naproxen [22204-53-1], $C_{14}H_{14}O_3$, and basic drugs such as disopyramide [3737-09-5], $C_{21}H_{29}N_3O$, tropicamide [1508-75-4], $C_{17}H_{20}N_2O_2$, atropine [51-55-8], $C_{17}H_{23}NO_3$, and homatropine [87-00-3], $C_{16}H_{21}NO_3$, have also been separated (21, 24). Table 3 lists some racemic compounds that have been completely resolved using a Chiral-AGP column (18). The AGP columns are also commonly used in the determination of enantiomers present at low concentrations in biological fluids such as plasma and urine (19, 25). Metoprolol was extracted from plasma and injected on a Chiral-AGP column for separation (25). It was possible to measure as little as 2 nmol/L plasma using fluorescence detection after separation.

1.2.4. Mechanism of Separation

Whereas the scientific basis of separation is well documented, ie, differences in binding between drug enantiomers and proteins, the mechanism for chiral recognition is not clearly understood. It is well known that the conformation of a native protein in solution can be altered by addition of organic modifiers and changes in pH. It is assumed that the AGP molecule has a high degree of flexibility even after bonding to the silica surface. Therefore adding modifiers to the mobile phase can alter the AGP molecule so that new chiral phases having different binding properties are induced (18, 21).

1.3. Bovine Serum Albumin Chromatographic Phases

1.3.1. Properties and Structure

Bovine serum albumin (BSA) is a globular protein having a molecular mass of 66,210. It consists of 581 amino acids in a single chain, and 17 intrachain disulfide bridges form nine double loops (26). Having an isoelectric point of 4.7, BSA is a relatively acidic protein. It is highly soluble in water, but like most globular proteins it precipitates from solution at high salt concentrations. BSA exhibits hydrophobic character and numerous examples of organic compounds binding to albumins have been reported (27). Whereas hydrophobic interactions contribute greatly to the total affinity of organic ligands for BSA, there are other contributions to consider, mainly electrostatic interactions, hydrogen bonding, and charge-transfer processes.

The first observation of the enantioselective properties of an albumin was made in 1958 (28) when it was discovered that the affinity for L-tryptophan exceeded that of the D-enantiomer by a factor of approximately 100. This led to more studies in 1973 of the separation of DL-tryptophan [54-12-6], $C_{11}H_{12}N_2O_2$, on BSA immobilized to Sepharose (29). After extensive investigation of the chromatographic behavior of numerous racemic compounds under different mobile-phase conditions, a BSA-SILICA hplc column (Resolvosil-R-BSA, Macherey-Nagel GmvH, Duren, Germany) was introduced in 1983.

Retention and stereoselectivity on the BSA columns can be changed by the use of additives to the aqueous mobile phase (30). Hydrophobic compounds generally are highly retained on the BSA, and a mobile-phase modifier such as 1-propanol can be added to obtain reasonable retention times. The retention and optical resolution of charged solutes such as carboxylic acids or amines can be controlled by pH and ionic strength of the mobile phase.

1.3.2. Applications

Various *N*-derivatives of amino acids (qv) are resolvable on BSA columns. These *N*-amino acid derivatives include benzenesulfonyl-, phthalimido-, 5-dimethylamino-1-naphthalenesulfonyl- (DANSYL-), 2,4-dinitrophenyl- (DNP-), and 2,3,6-trinitrophenyl- (TNP-) derivatives (30). Amines such as Prilocain, ()-2-(propylamino)-*o*-propiono-toluidide, a local anesthetic (Astra Pharm. Co.), are also resolved on BSA. The aromatic amino acids DL-tryptophan, 5-hydroxy-DL-tryptophan, DL-kynurenine [343-65-7], $C_{10}H_{12}N_2O_3$, and 3-hydroxy-DL-kynurenine [484-78-6], and drugs such as warfarin, phenprocoumon, and benzodiazepine derivatives can be separated on BSA as well.

1.4. Cellulose Triacetate and Cellulose Derivatives

1.4.1. Properties and Structure

Cellulose [9004-34-6] (qv) and other polysaccharides have long been known to have chiral recognition properties. Cellulose is readily available, inexpensive, and has good chemical stability. Microcrystalline cellulose triacetate [9012-90-3] (MCA), which is commercially available, is the product of the heterogeneous acetylation of microcrystalline cellulose (31). Various cellulose ester derivatives supported on macroscopic silica gel are available as hplc columns from Diacel (Diacel Chemical Industries, Ltd., Tokyo, Japan). These columns have good mechanical stability and mobile phases such as hexane–2-propanol or alcohols are used. The mechanism

of chiral recognition on cellulose-based phases is unknown, although hydrogen bonding and ligand inclusion play a part in resolution (32, 33) (see Cellulose esters).

1.4.2. Applications

MCA is used for the resolution of many classes of chiral drugs. Polar compounds such as amines, amides, imides, esters, and ketones can be resolved (34). A phenyl or a cycloalkyl group near the chiral center seems to improve chiral selectivity. Nonpolar racemates have also been resolved, but charged or dissociating compounds are not retained on MCA. Mobile phases used with MCA columns include ethanol and methanol.

The Diacel columns can be used for the separation of a wide variety of compounds, including aromatic hydrocarbons having hydroxyl groups, carbonyls and sulfoxides, barbiturates, and β-blockers (35, 36). There are presently nine different cellulose derivative-based columns produced by Diacel Chemical Industries. The different columns each demonstrate unique selectivities so that a choice of stationary phases is available to accomplish a separation.

1.5. Miscellaneous Chiral Chromatographic Techniques

Cyclodextrins are often used as chiral mobile-phase additives in miscellaneous chromatographic techniques as well as for stationary phases for thin-layer chromatography (35, 36). Gas chromatography (gc) columns consisting of derivatized cyclodextrins coated on the capillary column wall are also commercially available (Advanced Separation Technologies, Whippany, New Jersey). Chiral analytes that can be vaporized without degradation or racemization are suitable for analysis on the gc columns. Several reviews and books have been published on the various chiral chromatographic methods (35–37).

2. Biopolymers in Immunology

Biopolymers are employed in many immunological techniques, including the analysis of food, clinical samples, pesticides, and in other areas of analytical chemistry. Immunoassays (qv) are specific, sensitive, relatively easy to perform, and usually inexpensive. For repetitive analyses, immunoassays compare very favorably with many conventional methods in terms of both sensitivity and limits of detection.

2.1. Antigens

One condition that must be met for the application of an immunochemical method is that the analyte must be capable of stimulating an immune response leading to the formation of antibodies in the immunized animal. These antibodies can then be isolated and used as highly specific analytical reagents (immunoassays). Analytes that can combine with the corresponding antibodies are called antigens. There are physical and chemical restrictions on the types of analytes that may be used as immunoassay antigens. In general, large, rigid, chemically complex molecules make good antigens. For example, serum albumin, mol wt ca 60,000, is a good antigen because of its large size, complex structure, and good structural stability. Large repeating polymers such as lipids and carbohydrates, make poor antigens as a result of the simplicity of their structure, even though they may have high molecular weights and good structural stability. One molecule having mol wt 750, the smallest molecular mass that has demonstrated antigenic behavior, is *p*-azobenzene-arsonatetrityrosine (38).

A small analyte is not necessarily prevented from analysis by immunoassay, because a small molecule that is linked to a large antigenic molecule forms a new species having a modified surface structure. Small molecules made antigenic in this manner are called haptens. The larger molecule to which they are attached is called the carrier. The immune response to a hapten-carrier complex is actually triggered by two signals; one from the

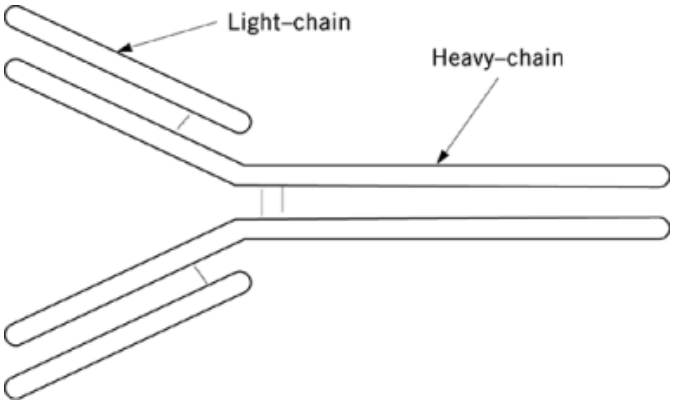

Fig. 4. Simple model of an IgG molecule showing light- and heavy-chain segments where a line (|) between the chains represents a disulfide bond.

hapten and one from its carrier. This is necessary, otherwise the hapten would provoke an immune response on its own. Even with the dual signal response, the signal triggered by the complex is still hapten-specific because the immune response is in part triggered by the hapten.

2.2. Antibodies

Antibodies are proteins, found in many body fluids such as tears, saliva, and urine, that are present in highest concentrations in blood serum. Because antibodies are proteins (qv), they may be characterized by such physical properties as solubility, electrostatic charge, isoelectric point, and molecular weight. The particular proteins which exhibit antibody activity are the immunoglobulins (Ig). The principal immunoglobulin in blood serum is immunoglobulin G (IgG), the structure of which is similar to the other immunoglobins. IgG is a glycoprotein having mol wt 150,000. It has a Y shape (38) and its structure is shown in Figure 4. There are estimated to be approximately 10 million potential combinations of antigen-binding specificities resulting from light- and heavy-chain combinations in the immunoglobulins. The possibility of utilizing all of these combinations as reagents in immunochemical methods is highly interesting though improbable.

2.3. General Methodology

2.3.1. Common Procedures

The general analytical scheme for immunochemical methods is rather simple. The analyte of interest, the antigen (Ag), reacts with the analytical reagent, the corresponding antibody (Ab), forming an immunochemical antigen–antibody complex:

$$\mathrm{Ag} + \mathrm{Ab} \rightleftharpoons \mathrm{Ag} - \mathrm{Ab}$$

The immunochemical interaction between the antigen and antibody is very specific. By labeling either the antigen or antibody, the method's sensitivity is increased. The most frequently used labels to increase sensitivity are radionuclides (see Radioisotopes) where the assay process is called radioimmunoassay (RIA), or enzymes where the assay is named enzyme immunoassay (EIA) (see Enzyme applications).

2.3.2. Labeling

Radioisotopic labeling, one of the first labeling methods used, is still prominent in assays where the use of nonradioisotopic labels has not been feasible (39). Labeling with enzyme to produce a spectrophotometrically detectable product is frequently used. Fluorescent labeling, used in conjunction with fluorescent polarization detection techniques, can be utilized for small molecules. A commercially available system uses this technique for monitoring drug levels in biological fluids (40). Rare-earth chelates such as those of europium [7440-53-1], Eu, are also successfully used as fluorescent labels (41). The rare-earth chelates have a large Stokes shift that helps to reduce interference from light scattering and from background fluorescence. In addition,these labels have fluorescent lifetimes lasting several hundred microseconds allowing delayed fluorescent sampling.

Chemiluminescent labels, in which the luminescence is generated by a chemical oxidation step, and bioluminescent labels, where the energy for light emission is produced by an enzyme-substrate reaction, are additional labeling types (39, 42). Luminol [521-31-3], $C_8H_7N_3O_2$, and acridine [260-94-6], $C_{13}H_9N$, derivatives are often used as chemiluminescent labels.

2.3.3. Variations in Methods

The various immunochemical methods can differ in a number of ways. For example, the analytical reagent may be crude antiserum, monoclonal antibodies, isolated immunoglobulin fractions, etc. The conditions under which the method is run, detection of the antigen–antibody complex, and the techniques used to increase sensitivity or specificity of the reaction all may be varied.

2.3.4. Heterogeneous and Homogeneous Assays

The various immunochemical techniques may be roughly divided into two groups. The first involves homogeneous procedures in which the separation of the bound and free labeled analyte, ie, the radionuclide or the enzyme, is not necessary. In contrast, heterogeneous immunochemical techniques require separation of the bound and free labeled analyte. The most common of these techniques is the enzyme-linked immunosorbent assay (ELISA) (43). In this technique either the antibody or the antigen (analyte) is attached to a solid phase, such as the walls of a polystyrene tube or surface of a plastic bead. Both a competitive and a double antibody (sandwich) ELISA technique are available for measuring antigens.

2.3.5. Sandwich Assays

In the sandwich technique, the most widely used ELISA, the solid surface is first coated with an appropriate antibody as shown in Figure 5. The sample solution containing the antigen (analyte) is then added and allowed to react with the bound antibody on the solid surface. After the reaction any remaining unbound antigens are washed away. Then an enzyme-labeled antibody, specific for a different site on the antigen, is added in a known amount for reaction with the bound antibody–antigen complex. After the reaction any unbound enzyme-labeled antibodies are washed away. A substrate is added which, when acted upon by the bound enzyme, produces a color change the amount of which is a direct measurement of specific enzyme-conjugated bound antibody and therefore of antigen present.

2.3.6. Competitive Assays

The next most widely used type of ELISA is the competitive assay (39, 43). In this technique the analyte of interest is mixed with a known amount of enzyme-labeled antigen and both compete for a limited number of binding sites on an antibody that is adsorbed on a solid support. After binding the excess free enzyme-labeled antigen or excess test antigen is washed away. Then a substrate is added which is acted upon by the enzyme-labeled antigen yielding a colored product. The amount of color development is proportional to the amount of enzyme-labeled conjugate bound to the antibody. Little or no color change indicates that the unlabeled antigen of interest was present in the test solution and was bound to the antibody. A color change indicates that

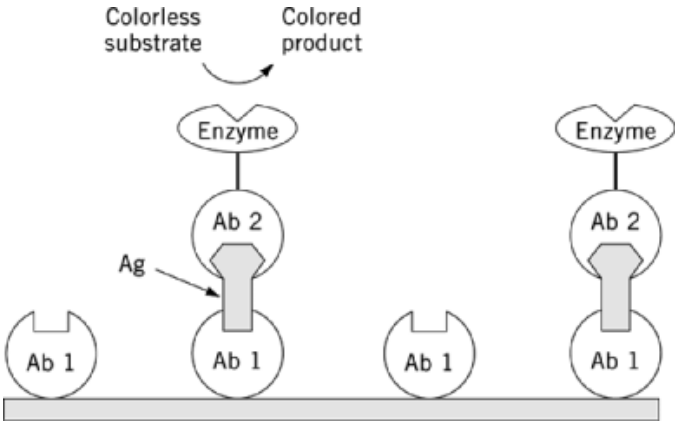

Fig. 5. Scheme of the last steps for the sandwich ELISA. Where Ab 1 represents the the surface-bound antibody, Ag, the antigen, and Ab 2, the enzyme-labeled antibody.

enzyme-labeled antigen was bound to the antibody, so that little or no unlabeled antigen was present in the original test solution.

2.4. Applications

Immunoassays are used in many different disciplines, having clinical, industrial, agricultural, and environmental applications. This technique has made possible rapid analysis of such varied analytes as viruses, toxins, hormones, foreign proteins, drugs, and insecticides.

As a result of regulations by the Food and Drug Administration, very sensitive and specific analysis techniques have been developed to test for food additives (qv) and possible contaminants in food, many of which are immunochemical methods. For example, botulism toxin and staphylococcal enterotoxins can be detected by ELISA. Animals for slaughter can be quickly screened for diseases such as brucellosis, tuberculosis, and cholera (43). The level of many regulatory enzymes in food can be monitored by immunoassays. Immunoassays are used in beer (qv) brewing to measure the levels of malt enzyme and amyloglucosidase, and in cheese production to measure the level of chymosin, microbial rennets, and beef pepsin [9001-75-6] (44). Immunoassays are also useful in the detection of foreign proteins added to foodstuffs. For example, the addition of cheaper meats, such as horse or kangaroo, into beef or pork products can be detected by immunoassays, as well as the inclusion of soybean protein in meat products. Immunochemical methods are often employed to control the production of gluten-free dietary products and hypoallergenic milk. Commercially produced ELISA immuno-kits for food analysis include tests for soya protein (Biokits Ltd., Clwyd, UK), meat species (Biokits Ltd., Clwyd, UK), papain [9001-73-4] (Labsystems Oy, Helsinki, Finland), *Salmonella* (Organon Teknika Corp., Durham, North Carolina), trichinosis (Agritech Systems, Portland, Maine; Idetek, San Bruno, California), aflatoxin B_1 [1162-65-8], $C_{17}H_{12}O_6$, (Neogen Corp., Lansing, Michigan; Agritech Systems, Portland, Maine; Immuno Systems, Biddeford, Maine; Biotech Research Lab, Rockville, Maryland; and Biokits Ltd., Clwyd, UK), aflatoxin M_1 [6795-23-9], $C_{17}H_{12}O_7$, (Neogen Corp., Lansing, Michigan), ochratoxin A (AFRC Food Research Institute, Norwich, UK; Biokits Ltd., Clwyd, UK), atrazine [1912-24-9] (Immuno Systems, Biddeford, Maine), antibiotic residues in milk (Angenics Inc., Cambridge, Massachusetts), zearalenone [17924-92-4], $C_{18}H_{22}O_5$, (Neogen Corp., Lansing, Michigan), and *Staphylococcus endotoxun* (Igen, Rockville, Maryland) (45).

Mycotoxins, toxic metabolites of some fungi, can be assayed by immunochemical techniques to determine concentration in animal feed and foodstuffs. Some of the analytes assayed in kits and the detection limits

Table 4. Detection Limits of Immunoassays Developed for Mycotoxins and Pesticides

Analyte	CAS Registry Number	Molecular formula	Method	Analyzed food	Detection limit, μg/kg[a]
		Mycotoxins			
aflatoxin B_1	[1162-65-8]	$C_{17}H_{12}O_6$	RIA, ELISA	peanuts, cereal	1–5
aflatoxin M_1			RIA, ELISA	milk	0.02–0.1[b]
ochratoxin A	[303-47-9]	$C_{20}H_{18}ClNO_6$	RIA, ELISA	cereals	0.2–2.0
zearalenone	[17924-92-4]	$C_{18}H_{22}O_5$	ELISA	cereals	20
		Pesticides			
parathion	[56-38-2]	$C_{10}H_{14}NO_5PS$	RIA	lettuce	10
diflubenzuron	[35367-38-5]	$C_{14}H_9ClF_2N_2O_2$	ELISA	milk	2
diclofop-methyl	[51338-27-3]	$C_{16}H_{14}Cl_2O_4$	EIA	sugarbeet, wheat, soybean	100–120
benomyl	[17804-35-2]	$C_{14}H_{18}N_4O_3$	RIA	fruit	500

[a] Detection limit is microgram of analyte per kilogram of sample unless otherwise noted.
[b] Units are microgram of analyte per liter of sample.

are listed in Table 4 (45). These assays are especially advantageous for routine analysis of large samples of foodstuffs (45, 46).

Immunochemical methods that utilize radioisotopic labeling can detect the use of anabolic sex hormones that increase the growth in meat animals. Stilbene [588-59-0], $C_{14}H_{12}$, trenbolone [10161-33-8], and zeranol [55331-29-8], $C_{18}H_{26}O_5$, can be successfully monitored by these immunoassay techniques (45). In order to prevent veterinary drugs from being transported to the human food chain, radioisotopic immunoassays were developed to monitor veterinary antibiotics such as penicillin and chloramphenicol [56-75-7], $C_{11}H_{12}Cl_2N_2O_5$, in meat, milk, and eggs (qv) (see Antibiotics; Meat products; Milk and milk products).

Pesticide contamination can also be monitored by ELISA. Immunoassays for pesticides are advantageous in that time-consuming sample preparation and purification steps can be avoided or reduced and very high specificity can be obtained in many cases. Various sample kits are available to assay plant tissues, soil, water, and biological fluids. Commercial kits are also available for the determination of atrazine, chlordane [57-74-9], $C_{10}H_6Cl_8$, heptachlor [76-44-8], $C_{10}H_5Cl_7$, aldicarb [116-06-3], $C_7H_{14}N_2O_2S$, aldicarb sulfone, glyphosate [1071-83-6], $C_3H_8NO_5P$, and chlorpyrifos [2921-88-2], $C_9H_{11}Cl_3NO_3PS$ (47).

Another use of immunoassays is in clinical diagnosis. Biological fluids such as serum and bronchial secretions can be assayed for indications of various disorders, including leukemia, carcinomas, and cancers. Some of the enzymes assayed and the corresponding disorders are listed in Table 5 (44). These assays are based mainly on changes in enzyme concentrations that result from enzyme release from injured tissue or from the changed metabolism of an affected organ. The goal in clinical diagnosis is to develop highly sensitive methods so that symptoms can be detected at the earliest possible stage.

3. Biopolymers as Biosensors

Selectivity is an important consideration in analytical chemistry. Biologically derived polymers can be used as highly selective immobilized reagents in analytical applications. The first reported use of immobilized biopolymers as biosensors (qv) for the detection of an analyte was made in 1962 (48). Since that first reported use there has been a great deal of development and application of immobilized biopolymers in analytical chemistry.

Table 5. Immunoassays of Enzymes in Clinical Diagnosis

Enzyme	Assayed medium	Detected disorder(s)
plasmin	serum	acute leukemia
pepsin, pepsinogen	serum	stomach ulcer, cancer, chronical gastritis
aspartate aminotransferase	serum	infarction, hepatitis
trypsin	serum	acute pancreatitis
pancreatic elastase	serum	pulmonar emphysema, acute pancreatic necrosis, arteriosclerosis
microbial elastase	bronchial secretions	chronical lung infections
acid phosphatase	serum	prostatic cancer
cystein proteinase	tumor medium	breast tumor
ribonuclease	serum	malignant diseases, renal failure
superoxide dismutase		lung cancer
glutamate-oxalacetate transferase	serum	differentiation of chronic liver disease
L-lactate dehydrogenase	serum	differentiation of heart cell damage

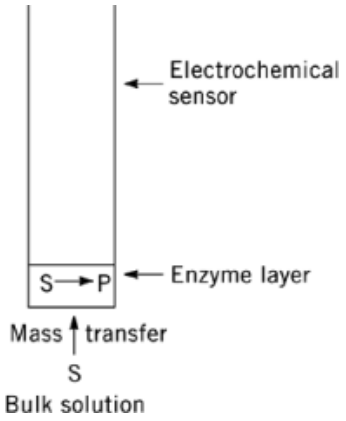

Fig. 6. Diagram of an immobilized enzyme electrode, where S is the substrate and P is the enzyme-bound substrate product.

3.1. Immobilized Enzymes

The immobilized enzyme electrode is the most common immobilized biopolymer sensor, consisting of a thin layer of enzyme immobilized on the surface of an electrochemical sensor as shown in Figure 6. The enzyme catalyzes a reaction that converts the target substrate into a product that is detected electrochemically. The advantages of immobilized enzyme electrodes include minimal pretreatment of the sample matrix, small sample volume, and the recovery of the enzyme for repeated use (49). Several reviews and books have been published on immobilized enzyme electrodes (50–52).

The enzyme can be immobilized on the electrode by several techniques (53). The simplest method, first used in 1962, is to trap an enzyme solution between the electrode surface and a semipermeable membrane. Another technique is to immobilize the enzyme in a polymer gel such as polyacrylamide which is coated on the electrode surface. Very thin-membrane films can be obtained by electropolymerization techniques (49, 54, 55) using polypyrrole, polyindole, or polyphenylenediamine films, among others. These thin films (qv) offer the advantage of improved diffusion of substrate and product that improves the response time. The enzyme can also be covalently bound to the electrode surface either directly or by using a small linking molecule (56). Covalent immobilization has the advantage of improving the stability of the enzyme (57).

The response of the immobilized enzyme electrode can be made independent of the enzyme concentration by using a large excess of enzyme at the electrode surface. The electrode response is limited by the mass transport of the substrate. Using an excess of enzyme often results in longer electrode lifetimes, increased linear range, reduced susceptibility to pH, temperature, and interfering species (58, 59). At low enzyme concentrations the electrode response is governed by the kinetics of the enzyme reaction.

3.1.1. Gas-Sensing Enzyme Electrodes

Potentiometry and amperometry are the most common electrochemical techniques to employ enzyme electrodes. Potentiometric gas-sensing and ion-selective electrodes have been converted into enzyme electrodes and used in various analytical applications (53). The gas-sensing electrodes for carbon dioxide and ammonia are most frequently converted to enzyme electrodes because of the lack of response to any dissolved ionic interferents. Decarboxylating or deaminating enzymes are immobilized to these gas-sensing electrodes so that the enzyme reaction product, CO_2 or NH_3, is detected. These potentiometric immobilized enzyme sensors are highly selective and are used for the detection of urea, creatinine, uric acid, amino acids (qv), and nucleotides, as well as other compounds (50, 53). Amperometric electrodes are generally coupled with oxidase or dehydrogenase enzymes. Oxidase enzymes can be immobilized on a Clark oxygen electrode and used to detect the amount of oxygen consumed in the enzyme reaction. For example, in the determination of creatinine in blood serum, the enzymes creatinine amidohydrolase, creatine amidinohydrolase, and sarcosine oxidase are coimmobilized on the polypropylene membrane of a Clark oxygen electrode (60). The enzymes catalyze the decomposition of creatinine with the consumption of oxygen, which is monitored by the Clark electrode. The oxidase enzymes can also be trapped on a platinum electrode and used to measure the amount of hydrogen peroxide produced in the enzyme reaction. For example, glucose oxidase [9001-37-0] covalently attached to platinum wire via glutaraldehyde [111-30-8], $C_5H_8O_2$, was used to determine glucose [50-99-7], $C_6H_{12}O_6$, levels by monitoring the amount of hydrogen peroxide formed (56). Dehydrogenase enzymes immobilized on glassy carbon or platinum electrodes are used to measure nicotinamide NADH or electron-transfer mediators such as ferrocyanide (53, 60). Amperometric immobilized enzyme electrodes are used in the determination of glucose and other sugars, amino acids, cholesterol [57-88-5], $C_{27}H_{46}O$, creatinine, uric acid, and alcohols (53, 60).

3.1.2. Multienzyme Electrodes

Coupling the reactions of two or more immobilized enzymes increases the number of analytes that can be measured. An electro-inactive component can be converted by an enzyme to a substrate that is subsequently converted by a second enzyme to form a detectable end product (57). For example, a maltose [69-79-4], $C_{12}H_{22}O_{11}$, sensor uses the enzymes glucoamylase and glucose oxidase, which convert maltose to glucose and then to gluconic acid [526-95-4], $C_6H_{12}O_7$, with the production of hydrogen peroxide, which is detected using a platinum anode (53).

Multienzyme electrodes can increase sensitivity from micromolar to nanomolar detection levels (53, 57). In this case the substrate is converted to a detectable product by one enzyme, then that product is recycled into the initial substrate by another enzyme resulting in an amplification of the response signal. For example, using lactate oxidase and lactate dehydrogenase immobilized in poly(vinyl chloride), an amplification of 250 was obtained for the detection of lactate (61).

3.1.3. Miscellaneous Biopolymeric Electrodes

Whereas immobilized enzyme electrodes are most commonly used in electrochemical systems, other types of biopolymers are also employed. For example, a catalytic biosensor uses immobilized antibodies capable of catalyzing chemical reactions (62). In this case, acidic products formed by the catalytic antibody are then detected electrochemically. A micro-pH electrode is used and the catalytic antibody is immobilized in a porous membrane. Biopolymers are also used as semipermeable electrode coatings. This use of biopolymers rejects

undesired interferents from the electrode-sensing surface while allowing the transport of the analyte. For example, cellulose acetate is used in this manner as a size-discrimination coating for electrodes (63, 64). Lipid coatings prevent polar electroactive compounds from reaching the electrode-sensing surface while letting hydrophobic substances pass through. Cast films of palmitic or stearic acids (65, 66) and phosphatidylcholine (67, 68) are also used for this purpose. A mixed lipid layer consisting of phosphatidylcholine and cholesterol has improved mechanical stability as well as permeable selective properties (61).

3.2. Enzyme Immunosensors

Enzyme immunosensors are enzyme immunoassays coupled with electrochemical sensors. These sensors (qv) require multiple steps for analyte determination, and either sandwich assays or competitive binding assays may be used. Both of these assays use antibodies for the analyte of interest attached to a membrane on the surface of an electrochemical sensor. In the sandwich assay type, the membrane-bound antibody binds the sample antigen, which in turn binds another antibody that is enzyme-labeled. This immunosensor is then placed in a solution containing the substrate for the labeling enzyme and the rate of product formation is measured electrochemically. The rate of the reaction is proportional to the amount of bound enzyme and thus to the amount of the analyte antigen. The sandwich assay can be used only with antigens capable of binding two different antibodies simultaneously (53).

In the competitive binding assay, sample antigen competes with enzyme-labeled antigen for the antibody binding sites on the membrane. The immunosensor is then placed in a solution containing the substrate for the labeling enzyme and the rate of reaction is measured electrochemically. The reaction rate is inversely proportional to the concentration of the sample antigen. This approach is applicable to many analytes including small hapten molecules such as therapeutic drugs.

Enzyme immunosensors are employed for the determination of Hepatitis B surface antigen, IgG, alpha-fetoprotein, estradiol, theophylline,insulin [9004-10-8], and albumin (69, 70). However, these immunosensors generally have slow response times and slow reversibility (57).

Enzyme immunosensors are used in flow injection systems and liquid chromatography to provide automated on-line analyses (71–73). These systems are capable of continuously executing the steps involved in the immunoassays, including the binding reactions, washing, and the enzyme reaction, in about 10 minutes.

4. Economic Aspects

Enantiomeric separations are expected to continue to have a considerable economic impact on the development of new drugs and therapy in the biomedical field. The Food and Drug Administration (FDA) has issued a set of guidelines on New Drug Applications in which the question of stereochemistry was approached directly for the manufacturing of drug substances (74). As of 1987, the FDA requires knowledge of the molecular structure of the drug substance. For chiral compounds this includes identification of all chiral centers. Whereas FDA guidelines do not discuss conditions under which a determination of absolute configuration is essential, it would be appropriate for supporting the manufacture of optically pure drugs (75). These requirements have led to a rapid growth in techniques that measure optical purity of drug substances. Listed in Table 6 are 1991 prices and manufacturers of chiral columns.

The importance of immunoassays for food monitoring and in the detection of diseases is expected to continue to grow as techniques and detection limits improve. In 1991, prices for most immunoassay kits ranged from $250 to $800 depending on the specific kit.

The development of biosensors is expected to benefit monitoring therapeutic drug levels, office testing, and implantable devices because of the advantages of cost-saving automation and data handling. A number of enzyme-based electrodes are commercially available and their manufacturers are given (69). Biosensors are

Table 6. Chiral Stationary Phases, Manufacturers, and Prices

Stationary phase	Manufacturer	Price[a], $
CYCLOBOND I, β-cyclodextrin	Advanced Separation Technologies Whippany, N.J.	425
CHIRAL-AGP, α_1-acid glycoprotein	Chrom Tech AB Norsburg, Sweden	1100
Enantiopac AGP, α_1-acid glycoprotein	LKB Products Bromma, Sweden	
RESOLVOSIL-BSA, bovine serum albumin	Macherey Nagel Germany	925
CHIRACEL-O series, cellulose derivatives	Diacel Chemical Industries Tokyo, Japan	1080

[a] Prices are from 1991 catalogs.

expected to become the predominant sensor technology by the year 2000. The largest market for biosensors is expected to be in clinical laboratories and drug monitoring (76) (see Automated instrumentation–clinical chemistry).

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