Biochemical Pharmacology, Vol. 32, No. 18, pp. 2647–2652, 1983.
Printed in Great Britain.

0006–2952/83 $3.00 + 0.00

COMMENTARY

IMMOBILIZED DRUGS AND ENZYMES IN BIOCHEMICAL PHARMACOLOGY

PERSPECTIVES AND CRITIQUE

LEMUEL B. WINGARD JR.
Department of Pharmacology, School of Medicine, University of Pittsburgh, Pittsburgh, PA 15261, U.S.A.

The immobilization of drugs and enzymes refers to their attachment to solid supports. In many cases the immobilized materials remain biologically active and can be used in numerous research investigations. The basic idea in immobilizing drugs or enzymes on solid supports is to control the gross movement of the immobilized material relative to that of the support. In most cases, the immobilized compound can still undergo conformational changes. If the compound is connected to the support through a spacer arm, then the compound can move several angstroms or micrometers relative to the support. However, the actual freedom of movement of immobilized materials depends on several factors, including (1) the type of immobilization chemistry employed, (2) the presence of interactions with the support, and (3) the use of single or multiple points of attachment as well as crosslinking between points on the immobilized material or between the immobilized material and the support. In this manner, drugs or enzymes can be retained in columns or other devices for action on solutions flowing through or in contact with the column or device. In a similar manner, immobilization can be used to retain drugs or enzymes in the medium surrounding small pore size inorganic particulates, polymer particles, organelles, or cells, and in this form used to study localized processes occurring in the surrounding medium. The immobilized agents also can be used in highly selective separation procedures based on affinity differences. The purpose of this commentary is to discuss actual and potential uses of immobilized drugs and enzymes in biochemical pharmacology, with emphasis on research methodology. Other applications of immobilization, such as the controlled release of immobilized drugs [1], the synthesis of drugs or of drug metabolites via immobilized enzyme systems [2, 3], and *in vitro* analytical chemistry techniques based on immobilized enzymes [4], are covered elsewhere.

The topics that are discussed in this commentary are: (1) immobilized drugs as a tool for drug mechanism studies, (2) immobilized drugs and enzymes in the development of electrochemical detectors for *in vivo* use, and (3) immobilized enzymes as a basis for artificial liver support.

Several dozen chemical techniques, plus many more variations, have been described for the immobilization of biochemicals on solid supports. These include trapping in gels or polymeric matrices, adsorption on surfaces, covalent attachment to organic and inorganic surfaces, and encapsulation in liposomes or other small vesicles. The detailed immobilization techniques are described elsewhere for enzymes [5, 6], affinity ligands [7], immunoproteins in radioimmuno or enzymeimmuno assays [8], microbial cells [9], solid phase peptide synthesis [10], and solid phase nucleotide synthesis [11]. Many of the same chemical methods for carrying out the above referenced coupling schemes have been or can be used for the immobilization of drugs on solid supports. A timely review listing specific drugs, hormones, and neurotransmitters and the supports on which they have been immobilized has been published by Venter [12]. The reader interested in learning if a particular drug has been immobilized, and on what support, is referred to that review [12].

The current interest in the immobilization of enzymes grew out of the early studies by Manecke in Germany and Katchalski in Israel in the early 1960's for coupling enzymes and antibodies to polymeric supports [13, 14]. A few years later, natural hormones such as adrenocorticotropic hormone (ACTH) and insulin were coupled covalently to agarose beads and tested for biological activity against isolated mammalian cells [15, 16]. However, later work suggested that there probably was considerable non-immobilized material present in the early studies with immobilized insulin [17]. Many other compounds have been immobilized on solid supports in the intervening years, so that by 1983 it is estimated that 400–500 different enzymes, 100 or more different drugs, and several hundred miscellaneous other compounds of biochemical interest (excluding agricultural pesticides, herbicides, and fertilizers) have been attached to solid supports with retention of various degrees of biological activity.

Immobilized drugs in mechanism studies

One of the perplexing problems in biochemical pharmacology is the development of methodology for assessing localized mechanisms of drug action

and the subsequent sequence of biochemical reactions that occur in organized heterogeneous cellular and subcellular structures. One approach is to use drugs labeled with radioactivity or with groups capable of producing intense fluorescence or unpaired electrons for spin labeling, incubating the labeled drug with cellular material, and then sorting out the cellular components to find out which parts contain bound drug. The problem is more complex when the drug can bind to several cellular components and, and in so doing, elicit more than a single action. The use of immobilized drugs is an important method for studying localized mechanisms of drug action in heterogeneous cellular systems. Conceptually the method seems quite simple. The drug is attached to a solid support and then incubated with the desired cellular or subcellular material. The presence and type of interaction are noted by determining the extent of binding, the influence on cell growth rates, the modification to subcellular rates of metabolism or functionality, or some other marker reaction. The practical implementation of such studies is much more difficult to conduct than appears at first glance. Scrupulous efforts must be made to free the preparation of adsorbed or otherwise non-bound drug and to measure the rate at which covalently bound drug is released. If the immobilized drug is still active, then it must be determined if the action is the same as that obtained with the non-immobilized form. These types of topics are addressed in this section with special relevance to (1) the use of immobilized drugs in probing drug actions on cell surface components, and (2) immobilized drugs in examining body fluid constituents.

Examples of the use of immobilized drugs and enzymes in probing the actions on cell surface components include several agents that bind to adrenergic receptors, some antitumor drugs, and enzymatic modification of cell surfaces. The interactions of immobilized isoproterenol (adrenergic agonist) and alprenolol (adrenergic β-blocker) with adrenergic receptors have been studied extensively using these drugs covalently attached to insoluble, and in the case of alprenolol also to soluble, supports [18, 19]. In both cases, the coupling chemistry was selected to give very stable binding, i.e. diazonium coupling with isoproterenol and a thioether linkage with alprenolol. This contrasts with less stable ester or amide linkages which may undergo hydrolysis to release drug at unacceptably high rates for immobilized drug studies. Acid washed isoproterenol-glass beads gave release rates of only 0.008%/hr at 20° [20], which was far too low to account for the observed biological effects when the immobilized preparation was placed in contact with isolated cardiac muscle strips. In general, coupling to agarose via cyanogen bromide activation gives less stable binding, as evidenced by a release rate of 1%/hr with catecholamines [21] and 2.5%/hr for lysine [22]. The points of attachment of the supports to isoproterenol and alprenolol are shown in Fig. 1. In both cases, the attachment was made at points that were generally acknowledged not to be necessary for the *in vivo* pharmacologic effect. Steric hinderance may be a factor in preventing the immobilized drug from fitting normally with a cell surface receptor site.

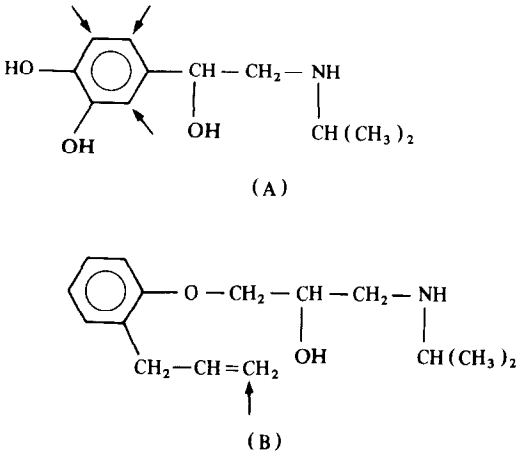

Fig. 1. Coupling positions, shown by arrows, for isoproterenol (A) and aprenolol (B). In (A) the attachment could occur at any of the arrows (occurs ortho or para to phenolic group).

Steric effects were demonstrated when alprenolol was coupled to 40,000 molecular weight dextran through spacer arms of 13, 8, and 4 atoms in length [23]; the resulting binding to frog erthyrocyte membrane β-receptors increased with increasing chain length.

To study the cell surface action of a drug that also has a direct intracellular action, it is necessary to prevent the drug from entering the cell. One method that has been used is to couple the drug, along with a marker material, to molecules that do not cross cell membranes. This approach was demonstrated by the covalent attachment of acridine orange, Hoechst 33258 dye, primaquine, mercury, and other compounds to radio iodinated or tritiated dextran of molecular weight 150,000 [24]. The argument was made that, since dextran did not cross cell membranes or bind to cell surfaces in appreciable amounts, then any interaction with the cell must have been attributable to the material coupled to the dextran. The author cited several convincing experiments to show that the method was reliable to assess binding to cell surface components. However, the studies involving incubation of the derivatized dextrans with murine leukemic cells did not show convincing evidence for the lack of soluble drug or for negligible cellular uptake of the dextran conjugate by endocytosis [24]. With soluble supports such as dextran, it is more difficult to measure the rates of release of drug or the rates of cellular uptake of support, as compared to insoluble supports, since, the insoluble support can be more completely separated from the solution containing the released drug or cells. However, the removal of adsorbed drug may be more of a problem with insoluble supports, especially if both the drug and the support have appreciable hydrophobic character. If the release rate of coupled and adsorbed drug can be reduced to acceptable levels and if the coupled drug shows biological activity, then it appears advantageous to use a solid (insoluble) support instead of a soluble support.

The anthracycline antitumor agents serve as an excellent example of a class of drugs that has an intracellular site of action and may also have a site of action on the cell surface. The use of insoluble supports currently is being used to differentiate the generally accepted intracellular mode of action from the postulated cell surface mode of action of some of the anthracycline agents. Adriamycin and carminomycin have been coupled covalently to polymeric supports and then shown to inhibit the survival of clones of cultured L1210 murine tumor cells [25, 26]. In the initial studies, the immobilized drugs were washed until no drug could be detected coming off of the support. In our current work we are using more sensitive methodology, which can detect release rates of 2–8 ng drug per day per g of support after several months of washing. This rate is far below the quantities of released drug needed to inhibit L1210 clones under the conditions used in the experiments. The strong binding of anthracycline compounds to many materials makes the release of free drug especially difficult to eliminate. An interesting experiment to show the absence of free drug would be to place a drug-permeable membrane between the immobilized drug and the cultured cells. Lack of evidence for modification of cell growth could be interpreted as a lack of free drug, but only if it could be demonstrated that released drug was not simply adsorbed on the membrane or on the walls of the container. In the anthracycline studies, it is also particularly important that the support particles be large enough to preclude any cellular uptake of immobilized drug by endocytosis. The possibility of such uptake is exemplified in a recent study whereby adriamycin was coupled to polyglutaraldehyde microspheres having an average diameter of 0.45×10^{-6} m and the particles incubated with L1210 cells [27]. Since L1210 cells typically are about 10×10^{-6} m in diameter, the possibility for cell uptake of immobilized drug via endocytosis could not be ruled out.

Immobilization at different points on the drug molecule is helpful in deciding whether the same mechanism of action applies to the immobilized as well as the free drug. Alternative attachment strategies may be easier to achieve with more complex molecules, such as the anthracycline agents, as compared to small molecular weight catecholamines since there are more functional groups present in the larger molecule that probably are not involved in producing the biological action.

It is also possible to modify the concentration of specific appendages attached to the surface of cells through the use of enzymes immobilized on insoluble supports. Easy and essentially complete separation of the modifying enzymes from the cells may be a distinct advantage in some experimental systems [28]. For example, immobilized exoglycosidases such as neuraminidase, β-galactosidase, α-mannosidase, and β-*N*-acetylglucoaminidase could be used in the stepwise removal of *N*-acetylneuraminic acid, galactose, mannose, and *N*-acetylglucosamine, respectively, from the glycoprotein appendages that dangle from the surface of many types of mammalian cells. Such modification to cell surface components should be of use in immunopharmacology studies. Immobilized lectins, such as concavalin A, are commercially available for affinity isolation of mannose-terminated oligosaccharides. The feasibility of using immobilized neuroamidase to modify the surface of mammalian cells was shown using *Vibrio cholerae* neuraminidase covalently coupled to the inside of 1.0 mm inner diameter nylon tubing; the immobilized enzyme released neuraminic acid from thymus and spleen lymphocytes of leukemic AKR mice when the cells were passed through the tube [29].

Immobilized drugs and enzymes also can play a major role in examining or modifying constituents in body fluids. Examples discussed here include heparin and related enzymes of the coagulation and lipolysis systems and perfusion of plasma from tumor-bearing animals over immobilized protein A.

The field of anticoagulation chemistry has been aided by the availability of methods for covalent immobilization of coagulation factors and anticoagulant agents onto solid supports. One example is the preparation of heparin coated surfaces. One of the main actions of heparin is to inhibit the enzymatic action of thrombin. This takes place by heparin activation of antithrombin III, which in turn leads to inhibition of thrombin. Covalent immobilization of heparin on polyvinyl alcohol showed retention of about 45% of the anticoagulant activity in the presence of antithrombin III with little evidence for release of bound anticoagulant [30]. Heparin also acts to release lipoprotein lipase from vascular beds into the general circulation. By immobilizing the lipoprotein lipase on a polymeric support, the specific binding sites for heparin and for a type of cofactor, apolipoprotein CII, with the enzyme could be worked out [31]. In addition, protein phosphorylation, using radiolabeled phosphorous, was studied in platelets stimulated by thrombin immobilized on a solid support [32]. This last example represents the use of an immobilized stimulant (thrombin) to trigger a platelet surface event that leads to the conduct of a critical intracellular process (phosphorylation).

Protein A, from the cell wall of *Staphylococcus aureus* Cowans 1, apparently is capable of inducing a modification to human or canine plasma that, in turn, causes a marked regression of breast adenocarcinoma [33, 34]. This preliminary conclusion was obtained when plasma from several patients, as well as test dogs, was perfused over protein A immobilized (presumably by adsorption) on a collodion charcoal matrix and then reinfused into the circulatory system of the subject. Protein A is known to react with the Fc region of mammalian immunoglobulins, especially IgG, and to combine with immune complexes in serum. The results of the infusions suggest that the contact of plasma with protein A produces a rapid elevation of tumor-specific antibodies which, in turn, account for the remission of the adenocarcinomas. The assessment of the role of the immobilized protein A, and what leakage rate of immobilized protein might be present, still remain to be reported [34, 35]. Similar studies in the treatment of neoplastic diseases have been carried out using either extracorporeal perfusion over beds of immobilized asparaginase or injection of entrapped asparaginase as a way of starving tumor cells incapable of synthesizing asparagine [36]; how-

ever, this approach does not look very promising at present.

Several other ideas for experimental applications of immobilizations are noted here. They may have been tried, but this author is not aware of reports of successful results. The binding of immobilized drugs to receptors is used extensively in affinity techniques for isolation of receptors; however, more definitive information on the binding sites might be obtained if the drug–receptor conjugate could be fixed by crosslinking the two participants together. Anchoring of cell fragments to solid supports may provide a method for studying the detailed reaction between a cell fragment component, such as the intracellular side of a receptor, and an exogeneous compound. The opposite case, i.e. an immobilized drug plus non-immobilized cell fragements and endogenous compounds, is suggested by recent studies of the interaction between calmodulin and immobilized phenothiazines [37]. And finally, the possibility of studying the transformation of cells while they are immobilized on a solid support is very appealing because the separation of cells from various growth or test reagents could be done so easily and completely. This possibility seems worth exploring since some types of animal cells can be immobilized on solid supports with retention of viability.

Immobilized enzyme/drug electrochemical detectors for in vivo *use*

Progress in biochemical pharmacology depends in large part on the availability of highly sensitive methods for *in vivo* chemical analysis of drugs, metabolites, and endogenous messenger compounds. However, in most present techniques, samples need to be removed from the organism under study and assayed *in vitro*. Over the last few years, several laboratories have been engaged in the development of electrochemical sensors for direct *in vivo* use in measuring concentrations of key compounds. The sensor specificity is obtained through immobilization of drugs or enzymes on the surface of a solid electrode. Two examples of such *in vivo* electrochemical sensors are discussed, one for glucose and the other for catecholamines.

The development of a sensor for continuous *in vivo* measurement of glucose concentrations is a high priority item for improving the treatment of insulin-dependent diabetes mellitus. Several groups have worked on a design that incorporated immobilized glucose oxidase for specificity and the net disappearance of oxygen for quantitation (see Ref. 38 for summary). The basic design, described by Updike *et al.* [39], was based on the measurement of the current produced by a Clark-type oxygen electrode (amperometric). However, problems in stability and linearity have hindered the clinical use of this sensor. An alternative immobilized glucose oxidase sensor has been described recently [40], wherein quantitation was obtained by amperometric measurement of the hydrogen peroxide generated by the enzyme-catalyzed reaction. Interference from ascorbic acid, biliburin, and uric acid was eliminated through use of a membrane permeable to hydrogen peroxide but impermeable to the above three oxidants. This sensor has performed well in animal studies. A possible third type of immobilized glucose oxidase sensor is based on the potentiometric response of the enzyme electrode to glucose [38, 41]. This third type of sensor is undergoing further *in vitro* evaluation to determine an electron conducting support (to which the enzyme is immobilized) that will provide a stable background potential.

The second area of immobilized enzyme/drug electrodes for *in vivo* use is that of neurochemistry. Much effort has been spent in trying to devise methodology for *in vivo* measurement of the levels of brain catecholamines. These materials readily undergo oxidation at a graphite electrode and can be quantified by measuring the current at the appropriate applied potential. However, the redox potentials of two of the most important compounds, dopamine and norepinephrine, are too close together to use voltametry to distinguish between these compounds. The problem is made worse by the fact that ascorbic acid also is oxidized at the same potential and, in addition, often is present in brain tissue at much higher levels than dopamine or norepinephrine. Therefore, it is encouraging to see several recent publications that suggest ways to overcome the ascorbic acid interference. Adams and co-workers [42] have immobilized ascorbic acid oxidase between two membranes that cover the tip of a graphite-Nujol voltametric electrode. The enzyme catalyzed the removal of the ascorbic acid from the vicinity of the voltametric electrode, so that the measured current was due only to oxidation of catecholamines. The authors are adapting this technique for use on 50–300 μm tip diameter carbon electrodes for implantation in intact animals. Another recent approach demonstrated a separation of about 0.21 V between the peak potentials for ascorbic acid and catechol when the catechol was immobilized on an α-alumina-modified glassy carbon electrode [43]. This shift in potential could be very useful in devising an *in vivo* catecholamine sensor. In still a third approach, oxidized apomorphine was adsorbed strongly on carbon paste-Nujol electrodes and catalyzed the oxidation of ascorbic acid [44], and again the peak potential for the ascorbic acid oxidation was shifted 0.35 V in the more negative direction. The apomorphine electrode did not shift the peak voltage for the oxidation of dopamine at pH 7, so this may be another way to separate the voltametric peaks for oxidation of dopamine and ascorbic acid.

Artificial liver support

In fulminant (acute) liver failure, significant increases in the plasma and sometimes spinal fluid concentrations of certain endogenous materials generally are observed. Phenols, ammonia (and usually glutamine), mercaptans, fatty acids, and sometimes other compounds reach concentrations which probably are toxic to hepatic, central nervous system, or other tissues [45]. However, there is considerable controversy over whether these compounds, i.e. phenols, ammonia, mercaptans, etc., are the principal causes of hepatic coma and associated encephalopathy or whether the pathological conditions might

be due instead to the inability of the liver to synthesize some unknown material. There is also some evidence that, in patients experiencing acute liver failure of viral hepatitis origin, the concentration of some middle molecular weight compounds, i.e. 1000–1500 daltons, increases significantly; these higher molecular weight compounds may be the more important as far as toxicity [46]. Therefore, if toxic compounds rather than the absence of a key compound is the governing factor in patient deterioration, then whether the toxins are small or middle molecular weight compounds is somewhat academic so long as the offending materials can be removed. Chang [47] has shown that the return of consciousness on recovery from hepatic coma probably is related to the removal of the middle molecular weight materials. However, there is very little evidence as to the identities of these middle molecular weight materials; and the experimental methodology used to remove larger materials also would remove smaller molecular weight phenols, mercaptans, loosely protein-bound compounds, and some amino acids [47].

Most of the experimental studies and clinical trials with extracorporeal perfusion of blood from hepatic comatose animals or patients have used perfusion over charcoal for removal of the toxic agents. The non-selective adsorption of plasma components on specially coated or encapsulated charcoal holds promise for improving the long-term recovery of patients from hepatic coma. But the results are far from conclusive. Perfusions through beds of ion exchange resins, albumin-coated agarose, or cultured liver cells also are methods that have been tried but for various reasons have not shown great promise so far. Thus, consideration has been given to extracorporeal perfusion over beds of immobilized enzymes as an alternative method for detoxification (and hopefully prevention of encephalography) of fulminant liver failure patients (with the expectation of liver regeneration taking hold for long-term recovery).

In normal hepatic tissue, the small molecular weight toxins, such as phenols, mercaptans, and ammonia, are metabolized to much less toxic agents for subsequent renal or biliary elimination. The enzyme systems that govern these and related detoxification reactions include the following: UDP-glucuronyl transferase for conjugation with glucuronic acid, phosphoadenosine 5′-phosphosulfate transferase for conjugation with sulfate, a variety of acyl thiokinase/transacylases for conjugation of glycine with organic acids, glutathione-*S*-aryl (or alkyl) transferase for formation of mercapturic acids, *S*-methyl transferase especially for methylation of mercaptans, mixed function oxidases or oxygenases (cytochrome P-450/NADPH/O_2 system) for a wide variety of oxidation reactions, and other enzymes that catalyze hydrolysis reactions or reduction reactions. Most of the conjugation enzymes listed above, with the exception of the sulfo and glutathione transferase, are membrane bound in the hepatocytes. However, methods have been developed for isolation and immobilization of several of these enzymes as well as for some of the cytochrome P-450 oxidation enzymes.

The immobilization of detoxification enzymes on solid supports so far has included covalent coupling and encapsulation. Brunner and co-workers [48–50] immobilized rabbit liver UDP-glucuronyl transferase by covalent coupling to CNBr-activated agarose. Using specially prepared large diameter agarose beads for minimizing hemolysis and platelet damage, they obtained loadings of 0.22 mg protein/mg dry agarose, which was equivalent to about 10 mg protein/ml of swollen packed gel. The stability of the immobilized enzyme was much greater than that of the soluble enzyme. Similar results were described by another laboratory for covalent immobilization of the same enzyme to Sepharose 4B type agarose [51, 52] at a loading of 3 mg protein/ml of packed swollen support. The immobilized enzyme activity had a half-life of 45 days at 4° and 1–2 days at 25°, as compared to less than 10 days for the soluble enzyme at 4°. The immobilized enzymes were active in the conjugation of phenols and other compounds. Glutathione-*S*-transferase from pig liver was covalently attached to the inner surface of cellulose acetate hollow fibers by periodate oxidation [53]. The immobilized glutathione-*S*-transferase was active with no loss of activity at 37° during the first 8–9 min; however, only 30% of the activity remained after sterilization by 2.5 Mrad of gamma radiation. Preliminary work also has been mentioned [54] on the immobilization of *S*-methyl transferase by coupling to CNBr-activated agarose and to an acrylic acid-acrylamide copolymer. Immobilization of several of the above enzymes also has been done at high loadings by encapsulation in liquid lipid membranes; however, this does not appear to be a useful technique for liver support perfusions because of the problem of preventing release of foreign enzymes during incubation of the liquid capsules with blood.

At least three reports have appeared on the immobilization of cytochrome P-450 and related detoxification systems. The mixed function oxidase, dimethylaniline monooxygenase, that catalyzes *N*- and *S*-oxidations has been coupled to nylon tubing and glass beads using glutaraldehyde, but the stability was fairly poor (half-lives of 5 hr to 10 min) [55]. Cytochrome P-450 and NADPH–cytochrome P-450 reductase were coupled to agarose, copolymerized with acrylic acidacrylamide, and encapsulated in liquid lipid membranes by one group [56] and co-immobilized with UDP-glucuronyl transferase on Sepharose by another group [57]. The immobilized materials were active and on Sepharose showed very useful activity at 37°.

REFERENCES

1. A. A. Sinkula, in *Sustained and Controlled Release Drug Delivery Systems* (Ed. J. R. Robinson), p. 411. Marcel Dekker, New York (1978).
2. J. Konecny, in *Enzyme Engineering* (Eds. G. B. Brown, G. Manecke and L. B. Wingard Jr.), Vol. 4, p. 253. Plenum Press, New York (1978).
3. C. Fenselau, S. Pallante and I. Parikh, *J. med. Chem.* **19**, 679 (1976).
4. L. B. Wingard Jr., E. Katchalski-Katzir and L. Goldstein (Eds.), *Applied Biochemistry and Bioengineering*, Vol. 3. Acadamic Press, New York (1981).

5. K. Mosback (Ed.), *Methods in Enzymology*, Vol. 44. Academic Press, New York (1976).
6. L. Goldstein and G. Manecke, in *Applied Biochemistry and Bioengineering* (Eds. L. B. Wingard Jr., E. Katchalski-Katzir and L. Goldstein), Vol. 1, p. 23. Academic Press, New York (1976).
7. W. B. Jakoby and M. Wilchek (Eds.), *Methods in Ezymology*, Vol. 34. Academic Press, New York (1974).
8. J. L. Guesdon and S. Avrameas, in *Applied Biochemistry and Bioengineering* (Eds. L. B. Wingard Jr., E. Katchalski-Katzir and L. Goldstein), Vol. 3, p. 207. Academic Press, New York (1981).
9. J. Klein and F. Wagner, in *Applied Biochemistry and Bioengineering* (Eds. I. Chibata and L. B. Wingard Jr.). Vol. 4, p. 11. Academic Press, New York (1983).
10. J. M. Stewart and J. D. Young, *Solid Phase Peptide Synthesis*. W. H. Freeman, San Francisco (1969).
11. M. D. Matteucci and M. H. Caruthers, *J. Am. chem. Soc.* **103**, 3185 (1981).
12. J. C. Venter, *Pharmac. Rev.* **34**, 153 (1982).
13. G. Manecke, *Pure appl. Chem.* **4**, 507 (1962).
14. I. H. Silman and E. Katchalski, *A. Rev. Biochem.* **35**, 873 (1966).
15. B. P. Schimmer, K. Veda and G. H. Sato, *Biochem. biophys. Res. Commun.* **32**, 806 (1968).
16. P. Cuatrecasas, *Proc. natn. Acad. Sci. U.S.A.* **63**, 450 (1969).
17. M. B. Davidson, A. J. VanHerle and L. E. Gerschenson, *Endocrinology* **92**, 1442 (1973).
18. J. C. Venter and C. M. Fraser, *Fedn Proc.* **42**, 273 (1983).
19. J. Pitha and J. W. Kusiak, *Fedn Proc.* **42**, 279 (1983).
20. J. C. Venter, L. J. Arnold Jr. and N. O. Kaplan, *Molec. Pharmac.* **11**, 1 (1975).
21. G. Vanquelin, M. L. Lacombe, J. Hanoune and A. D. Strossberg, *Biochem. biophys. Res. Commun.* **64**, 1076 (1975).
22. M. Wilchek and T. Miron, *Biochem. Intern.* **4**, 629 (1982).
23. J. Pitha, J. Zjawiony, R. J. Lefkowitz and M. C. Caron, *Proc. natn. Acad. Sci. U.S.A.* **77**, 2219 (1980).
24. J. Pitha, *Eur. J. Biochem.* **82**, 285 (1978).
25. T. R. Tritton and G. Yee, *Science* **217**, 248 (1982).
26. T. R. Tritton, G. Yee and L. B. Wingard Jr., *Fedn Proc.* **42**, 284 (1983).
27. Z. A. Tokes, K. E. Rogers and A. Rembaum, *Proc. natn. Acad. Sci. U.S.A.* **79**, 2026 (1982).
28. L. B. Wingard Jr., in *Enzyme Engineering Future Directions* (Eds. L. B. Wingard Jr., I. V. Berezin and A. A. Klyosov), p. 313. Plenum Press, New York (1980).
29. E. R. Bazarian and L. B. Wingard Jr., *J. Histochem. Cytochem.* **27**, 125 (1979).
30. Y. Muira, S. Aoyagi, Y. Kusada and K. Miyamoto, *J. biomed. Mater. Res.* **14**, 619 (1980).
31. N. Matsuoka, K. Shirai and R. L. Jackson, *Biochim. biophys. Acta* **620**, 308 (1980).
32. W. C. Wallace and H. B. Bensusan, *J. biol. Chem.* **255**, 1932 (1980).
33. D. S. Terman, J. B. Young and 15 other co-authors, *New Engl. J. Med.* **305**, 1195 (1981).
34. D. S. Terman, J. B. Young, W. T. Shearer, C. Avers, D. Lehane, H. I. Zaleski and Y. Daskal, *New Engl. J. Med.* **306**, 937 (1982).
35. T. M. S. Chang, *New Engl. J. Med.* **306**, 936 (1982).
36. P. Edman and I. Sjoholm, *J. pharm. Sci.* **71**, 576 (1982).
37. C. Rochette-Egly, E. Boschetti, P. Basset and J-M. Egly, *J. Chromat.* **241**, 333 (1982).
38. L. B. Wingard Jr., *Fedn Proc.* **42**, 288 (1983).
39. S. J. Updike, M. Schultz and B. Ekman, *Diabetes Care* **5**, 207 (1982).
40. L. C. Clark Jr. and C. A. Duggan, *Diabetes Care* **5**, 174 (1982).
41. L. B. Wingard Jr., C. C. Liu, S. K. Wolfson Jr., S. J. Yao and A. L. Drash, *Diabetes Care* **5**, 199 (1982).
42. G. Nagy, M. E. Rice and R. N. Adams, *Life Sci.* **31**, 2611 (1982).
43. J. Zak and T. Kuwana, *J. Am. chem. Soc.* **104**, 5514 (1982).
44. H. Y. Cheng, E. Strope and R. N. Adams, *Analyt. Chem.* **51**, 2243 (1979).
45. L. Zieve, in *Proceedings of the International Symposium on Artificial Liver Support* (Eds. R. Williams and I. M. Murray-Lyon), p. 11. Pitman Medical, Kent (1975).
46. H. W. Leber, J. Klausmann, G. Goubeaud and G. Schutterle, in *Artificial Liver Support* (Eds. G. Brunner and F. W. Schmidt), p. 96. Springer, Berlin (1981).
47. T. M. S. Chang, in *Artificial Liver Support* (Eds. G. Brunner and F. W. Schmidt), p. 126. Springer, Berlin (1981).
48. G. Brunner, in *Proceedings of the International Symposium on Artificial Liver Support* (Eds. R. Williams and I. M. Murray-Lyon), p. 153. Pitman Medical, Kent (1975).
49. G. Brunner, C. J. Holloway and H. Losgen, *Int. J. artif. Organs* **2**, 163 (1979).
50. H. Losgen, G. Brunner, C. J. Holloway, B. Buttelmann, S. Husmann, P. Scharff and A. Siehoff, *Biomat. med. Dev. art. Org.* **6**, 151 (1978).
51. I. Parikh, D. W. MacGlashan and C. Fenselau, *J. med. Chem.* **19**, 296 (1976).
52. C. Fenselau, S. Pallante and I. Parikh, *J. med. Chem.* **19**, 679 (1976).
53. L. Callegaro, A. Boniolo and A. Malinverni, in *Artificial Liver Support* (Eds. G. Brunner and F. W. Schmidt), p. 202. Springer, Berlin (1981).
54. G. Brunner, in *Artificial Liver Support* (Eds. G. Brunner and F. W. Schmidt), p. 230. Springer, Berlin (1981).
55. L. L. Poulsen, S. S. Sofer and P. M. Ziegler, *Meth. Enzym.* **44**, 849 (1976).
56. G. Brunner, H. Losgen, B. Gawlik and K. Belsner, in *Biochemistry, Biophysics and Regulation of Cytochrome P-450* (Eds. J. A. Gustafsson, J. Carlstedt-Duke, A. Mode and J. Rafter), p. 573. Elsevier/North Holland Biomedical Press, Amsterdam (1980).
57. J. P. Lehman, L. Ferrin, C. Fenselau and G. S. Yost, *Drug Metab. Dispos.* **9**, 15 (1981).