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Volume I

Immobilized Enzyme Principles

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Immobilized Enzyme Principles

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Introduction to the Series

The biological sciences have made remarkable progress during the last two decades. A wealth of information has accumulated on the structure and function of the materials comprising the living organism, on the chemical and physical aspects of a great number of biological processes, on catalysis in biological systems, and on the relationship between structure and function in enzymes and other biospecific macromolecules. This work on fundamentals has been accompanied by salient achievements in the fields of microbial genetics, tissue culture, and related areas. Nonetheless, the communication gap between pure and applied science has still to be narrowed to make better use of the potential of some of the recent discoveries in biology. The cooperation and mutual esteem and understanding between basic scientists and engineers is thus needed to attain cross-fertilization between the diverse approaches and experiences of the two disciplines; hence it is the aim of this publication series to bring together comprehensive summaries of work being done in the overlapping areas of engineering and biology.

Several areas of interaction between biological scientists and engineers have already begun to emerge; the term bioengineering has been coined to cover this range of interactions. Chemical engineers and microbiologists have been working together in the industrial production of foods, beverages, and chemicals by fermentation. Mechanical engineers and chemical engineers have cooperated with physiologists and people in the medical sciences to develop artificial organs, special life-support machines, artificial materials, and prosthetic devices. Electrical engineers together with physiologists have begun to apply system approaches to the study of biological control mechanisms. More recently biochemists and biophysicists have interacted with chemical engineers to explore the utilization of enzymes as special catalysts for use in industrial processing, analytical chemistry, and medicine.

The basic understanding of biological phenomena appears rooted in events that occur at the molecular level. Since current biological research is heavily committed in this direction, it seems logical to stress

the underlying common denominator that biochemistry can bring to the understanding of the many facets of biological systems.

Thus the title of this serial publication, *Applied Biochemistry and Bioengineering,* has been selected to emphasize the biochemical common denominator underlying the interaction of engineering practice and the biological sciences for technological development. It is hoped that the series will provide guidance in the application of these technological developments for the benefit of mankind.

THE EDITORS
February 1976

Preface

Utilization of immobilization techniques for the study and application of enzyme catalysts in a variety of potential end uses seems to be an especially appropriate subject for the first volume of this series. Both scientific and engineering inputs are required, with a strong reliance on basic biochemistry and biophysics. This volume places a major emphasis on the preparation of enzyme-support systems, on the effects caused by the concurrent phenomena of enzyme-catalyzed reaction kinetics and mass transfer resistances, and on how these are incorporated into the design of enzyme-catalyzed reactor systems. An additional chapter is included to show some examples of the practical application of immobilized enzymes.

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Immobilized Enzymes—A Survey[1]

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Until rather recently, immobilized enzymes were more of a curiosity, conceived in the initial stages of the rapprochement between biochemistry and polymer chemistry. The motivations underlying the early activities could be rationalized as an attempt to apply the accumulated experience in "making" macromolecules to the more exacting task of grafting a biocatalyst onto a polymeric structure designed to lead to a biologically active conjugate. From the biochemist's point of view, such conjugates could serve as water-insoluble highly specific reagents, easily removable from the reaction mixture at any predetermined stage of the reaction.

This approach contained the seeds of essentially all basic concepts and developments that materialized in the decade that followed the preparation of the first stable and reusable water-insoluble enzyme derivatives in the late 1950s. Hence, realization of the potential of immobilized enzymes as a new type of model system for the investigation of isolated aspects of complex biological phenomena on the one hand, and of their industrial potential as a new type of highly specific heterogeneous catalyst for continuous processes on the other, brought together chemical engineers, organic and physical chemists, biochemists, biologists, and microbiologists, each with his own expertise. This meeting of disciplines, within the loose framework of "enzyme engineering" has generated new concepts as well as new technologies.

[1] The authors dedicate this article to Georg Manecke on his sixtieth birthday.

Historically, the earliest reported cases of protein immobilization involved physical adsorption of the protein onto particles such as charcoal, kaolinite, red blood cell stroma, cellulose, and glass beads (Nelson and Hitchcocks, 1921; Langmuir and Schaefer, 1938, 1939). The first attempts to make use of such preparations were soon to follow, and already in the 1930s work on the application of adsorbed antigens for the isolation of specific antibodies could be found in the immunological literature (for review, see Isliker, 1957). The unpredictable behavior of these systems and the inability to obtain clean separations led the early investigators to the realization that fixation by forces stronger than adsorption was necessary. It is thus not surprising that the initial attempts at covalent fixation onto water-insoluble supports were carried out by the immunologists. Landsteiner and Van der Scheer (1936) described the coupling of diazotized haptens to blood cell stroma, and the utilization of the insoluble preparations for the isolation of the corresponding antibodies. Their work was followed, after the interruption of World War II, by the first experiments on covalent binding of a variety of proteins including enzymes as well as antigens to chemically well defined water-insoluble polymeric supports. The methodology of binding, however, was limited to the commercial polymers available at the time—derivatized celluloses and styrene polymers. In 1949, Micheel and Evers described the covalent binding of proteins to carboxymethyl cellulose azide. Campbell *et al.* (1951) reported on the coupling of ovalbumin to diazotized *p*-aminobenzyl cellulose, and the isolation of ovalbumin antibodies on the immunoadsorbent thus obtained.

These first steps were soon followed by other methods of coupling proteins to polymers. Isliker (1953) prepared immunoadsorbents with carboxychloride and sulfonylchloride derivatives of polystyrene; Manecke (Manecke and Gillert, 1955) utilized diazotized poly(*p*-aminostyrene) and later a poly(4-isocyanatostyrene) derivative (Manecke *et al.*, 1958) for the same purpose. In parallel the immobilization of enzymes by similar approaches was tried by Grubhofer and Schleith (1953, 1954), who coupled carboxypeptidase and amylase to diazotized poly(*p*-aminostyrene) and by Manecke (Manecke and Gillert, 1955; Manecke *et al.*, 1958; Manecke and Singer, 1960) and Brandenberger (1955, 1956, 1957), who used poly(*p*-aminostyrene) and poly(4-isocyanatostyrene) to bind enzymes. The amounts of bound protein and the enzymic activities retained in the immobilized preparations obtained by these methods were, however, relatively poor, presumably owing to the hydrophobicity of the supports. This early work was improved upon by Mitz and Summaria (1961), who

coupled trypsin and chymotrypsin to *p*-aminobenzyl cellulose and carboxymethyl cellulose hydrazide preparations of known degrees of substitution, and by Katchalski (Bar-Eli and Katchalski, 1960, 1963; Cebra *et al.*, 1961; Katchalski, 1962), who prepared water-insoluble derivatives of trypsin and papain by coupling the enzymes to diazotized leucine–*p*-aminophenylalanine copolymers. In the case of trypsin, a polytyrosyl derivative of the enzyme was used to protect it from inactivation in the course of the coupling reaction. Concurrently a series of copolymers of methacrylic acid and methacrylic acid-3-fluoro-4,6-dinitroanilide of varying ratios of comonomers were prepared by Manecke (Manecke and Singer, 1960; Manecke, 1962). In these preparations the 3-fluoro-4,6-dinitroanilide group served as the reactive moiety, and the carboxylic groups as the component bestowing hydrophilicity.

This work was paramount in delineating the objectives as well as the problems facing the chemist aiming at the covalent immobilization of biologically active proteins. The main conclusions to be drawn were as follows: (1) Derivatized polymers with groups of different chemical specificities are needed for attaining biologically active immobilized preparations of different proteins. (2) The chemical nature of the support material may determine not only the amount of bound protein, but also the extent to which its biological activity is retained; more specifically, supports rich in hydrophobic groups give immobilized preparations of low stability while the presence of hydrophilic groups enhances the stability and may in some cases counteract the deleterious effects of a hydrophobic environment. (3) Protection of the enzyme by chemical modification prior to coupling may sometimes be necessary.

These ideas, summarized in several reviews (Manecke, 1962; Katchalski, 1962; Silman and Katchalski, 1966), led to more coherent attempts at designing polymers of predetermined characteristics in terms of their mechanical properties, their effect on the stability of the bound protein and the type of functional group through which they would attach to the protein. Thus the leucine-*p*-aminophenylalanine and methacrylic acid–methacrylic acid-3-fluoro-4,6-dinitroanilide copolymers were soon followed by ethylene-maleic anhydride copolymers (Levin *et al.*, 1964; Goldstein *et al.*, 1964), derivatized cellulose (Lilly *et al.*, 1965, 1966; Kay and Lilly, 1970), cyanogen bromide-activated Sephadex and Sepharose (Axén *et al.*, 1967; Porath *et al.*, 1967), and, somewhat later, derivatized acrylic polymers and copolymers (Inman and Dintzis, 1969; Mosbach, 1970; Barker *et al.*, 1970; Manecke *et al.*, 1970), derivatized porous glass (Weetall, 1969, 1970),

and derivatized nylons (Hornby and Filippusson, 1970; Inman and Hornby, 1972; Goldstein *et al.*, 1974; Campbell *et al.*, 1975). The work on enzyme immobilization has been extensively reviewed (Goldman *et al.*, 1971b; Melrose, 1971; Smiley and Strandberg, 1972; Orth and Brümmer, 1972; Royer *et al.*, 1973; Zaborsky, 1973; Manecke, 1974; Goldstein and Manecke, this volume).

The work of Inman and Dintzis (1969) on derivatized polyacrylamide suggested a more general approach to the problems of enzyme immobilization, i.e., the use of "parent carrier polymers," which can by consecutive chemical manipulations be transformed into the chemical species suitable for a specific task.

Although the mainstream in the methodology of enzyme immobilization centered until recently on covalent linking, considerable effort was devoted throughout the years to developing methods for noncovalent fixation of enzymes. Such methods would be more general, and of particular significance in the case of enzymes sensitive to chemical modification. The methods that have eventually gained acceptance are: physical adsorption of enzymes or enzyme derivatives onto supports of superior adsorptive properties, mainly ion exchangers (Tosa *et al.*, 1966; Messing, 1975; Hofstee and Otillio, 1973; Stanley and Palter, 1973; Gladishev *et al.*, 1973; Solomon and Levin, 1974); occlusion into cross-linked polymer gels (Bernfeld and Wan, 1963; Mosbach and Mosbach, 1966; Bernfeld *et al.*, 1968; Mosbach, 1970); and recently encapsulation into microcapsules (Chang, 1964, 1972; Chang *et al.*, 1966), fibers (Dinelli, 1972; Marconi *et al.*, 1974), and liposomes (Gregoriadis *et al.*, 1971; Gregoriadis, 1974).

The idea that the specificity of biological macromolecules as reflected in their high binding constants for substrates, inhibitors, or effectors, can be used for separation and purification through formation of insoluble complexes had been in the air since the early work on immunoadsorbents (Campbell *et al.*, 1951; Lerman, 1953a,b). This concept applied by Schramm and co-workers to the purification of α-amylase by precipitating the enzyme–substrate complex out of solution (Schramm and Loyter, 1962, 1966; Levitzki *et al.*, 1964), gained in importance with the perfection of immobilization techniques.

Immobilized derivatives of trypsin and chymotrypsin were used for the selective adsorption of the pancreatic inhibitors of these enzymes from crude extracts. The inhibitors were subsequently eluted under conditions where binding was weakest (Fritz *et al.*, 1966, 1967, 1968, 1969). The reversal of the procedure, i.e., the use of the purified inhibitors in immobilized form for the isolation of pure enzymes, was a natural extension of the same basic concept. The immense importance

of enzyme purification by selective adsorption was recognized soon thereafter in the report by Cuatrecasas, Wilchek, and Anfinsen (1968) on the *affinity chromatography* purification of staphylococcal nuclease, chymotrypsin, and carboxypeptidase A on columns containing synthetic low-molecular-weight inhibitors of these enzymes, covalently attached to a solid matrix. The method has found application in the purification of a wide variety of biological substances (for reviews, see Cuatrecasas and Anfinsen, 1971; Cuatrecasas, 1972; Wilchek, 1974; Dunlap, 1974; Jakoby and Wilchek, 1974; Wilchek and Hexter, 1976).

As was shown recently, immobilized analogs of cofactors, such as adenosine 5′-monophosphate (AMP), adenine nicotinamide dinucleotide (NAD^+) and pyridoxal 5′-phosphate, which have affinity for a broad spectrum of enzymes, could be used to adsorb an entire family of enzymes, individual members being then eluted by appropriate "specific elution" procedures (Mosbach, 1974; Mosbach *et al.*, 1971, 1972; Kaplan *et al.*, 1974). These advances in "general ligand" affinity chromatography cross-fertilized the field of immobilized enzymes, instigating methods for the fixation of enzymes via or together with immobilized cofactors or cofactor analogs. Some current work could illustrate these new trends: Enzymically active water-insoluble glycogen-phosphorylase *b* could be prepared by immobilizing the enzyme on an insoluble derivative of its effector, AMP[N^6-(6-aminohexyl)adenosine 5′-phosphate Sepharose] (Mosbach and Gestrelius, 1974). By a similar approach Fukui *et al.* (1975) immobilized tyrosinase and tryptophanase on water-insoluble derivatives of pyridoxal 5′-phosphate—an effector of these enzymes; their work furthermore demonstrated that, in the case of multisubunit enzymes, attachment to an insoluble matrix, containing immobilized effector, via site-directed binding to one subunit only, could be sufficient to attain immobilization (Fukui *et al.*, 1975).

Most of the initial work on the methodology of enzyme immobilization was done with hydrolases, in particular proteases, owing to their accessibility and relative simplicity. Insoluble derivatives of papain, trypsin, and chymotrypsin using different types of support materials, charged as well as electrically neutral, were thus among the first immobilized enzymes to be employed in the next phase of development, clarification of some of the more fundamental aspects of the kinetic behavior of immobilized enzymes and the first serious attempts to apply immobilized enzymes in the laboratory, as stable reusable and removable reagents. Work in these areas has been extensively reviewed and will be only briefly highlighted here (see Goldstein and

Katchalski, 1968; Goldstein, 1969, 1970; Stark, 1971; Laidler and Sundaram, 1971; Goldman *et al.*, 1971a,b; Katchalski *et al.*, 1971; Lilly and Dunnill, 1971, 1972; Vieth and Venkatasubramanian, 1973, 1974; Zaborsky, 1973; Bunting and Laidler, 1973; Weetall, 1975).

In 1964 Goldstein and co-workers showed that the pH-activity profiles of polyanionic derivatives of trypsin and chymotrypsin were displaced toward more alkaline pH values relative to the native enzymes; conversely, the pH-activity profiles of polycationic derivatives of the same enzymes were displaced toward more acidic pH values (Goldstein *et al.*, 1964; Goldstein and Katchalski, 1968; Pecht and Levin, 1972; Goldstein, 1972; Manecke, 1975). These effects could be interpreted in terms of changes in the local concentration of hydrogen and hydroxyl ions in the domain of the bound enzyme, i.e., by a modified microenvironment, due to Donnan-type partitioning of hydrogen ions between the bulk solution and the charged enzyme particles. The polyelectrolyte nature of these effects could be demonstrated by their cancellation at high ionic strength.

In the case of charged substrates (e.g., the systems, esters or amides of arginine, acted upon by polyanionic or polycationic derivatives of trypsin, bromelain, ficin, or papain), partitioning of substrate resulting from attractive or repulsive interactions with the polyelectrolyte support, i.e., higher or lower local substrate concentration, could account for the observed lowering or increase in the values of the apparent Michaelis constants (Goldstein *et al.*, 1964; Wharton *et al.*, 1968). Assuming a Boltzmann distribution for the charged low-molecular-weight species in solution, Goldstein *et al.* (1964) could relate the observed shifts in pH-activity curves (ΔpH) and Michaelis constants (ΔpK_m) to the electrostatic potential in the domain of a charged enzyme particle. These phenomena were later analyzed in greater detail (Wharton *et al.*, 1968; Hornby *et al.*, 1968; Shuler *et al.*, 1972; Sundaram *et al.*, 1970; Bunting and Laidler, 1973; Kobayashi and Laidler, 1973). It should be noted that some of the findings of Goldstein *et al.* (1964) were anticipated by McLaren (McLaren and Estermann, 1957; Esterman *et al.*, 1959; McLaren and Babcock, 1959; McLaren, 1960; McLaren and Packer, 1970), who reported alkaline shifts in the pH-activity curves of chymotrypsin adsorbed on kaolinite particles and ascribed the changes to differences in the surface pH of the particles. These authors were also the first to point out the biological implications of the observed phenomena. Their work, however, coming too early, and addressed to a different audience—the soil chemist interested in clays—remained relatively unknown until the mid-1960s.

The main conclusion to be drawn from these studies was that the magnitude of the perturbation of the apparent kinetic parameters of an immobilized enzyme could serve in principle as a measure of the effective concentrations of substrate, modifier, or inhibitor at the site of the enzymic reaction. Moreover, the microenvironment concept emphasized the uncertainties and limitations of the prevalent approach of reconstituting metabolic pathways in cells via solubilization of the multienzyme complexes and the study of individual enzymic reactions *in vitro* (see, e.g., Green and Silman, 1967; Brown, 1971; Katchalski *et al.*, 1971). These aspects gained in significance after the preparation of enzyme membranes (Goldman *et al.*, 1965, 1968a,b, 1971a,b; Selegny *et al.*, 1968; Broun *et al.*, 1969), enzyme columns (Bar-Eli and Katchalski, 1963; Lilly *et al.*, 1966; Lilly and Sharp, 1968; Hornby *et al.*, 1968), and immobilized multienzyme systems (Mosbach and Mattiasson, 1970; Mattiasson and Mossbach, 1971; Goldman and Katchalski, 1971; Broun *et al.*, 1972; Lecoq *et al.*, 1975).

In 1965 Goldman and co-workers found that a papain–collodion membrane acting on ester substrates displayed distorted pH-activity profiles (Goldman *et al.*, 1965, 1968a). The anomalies were attributed to the local accumulation of hydrogen ions, produced by the hydrolysis of the ester substrates within the porous membrane. This interpretation was supported by the finding that grinding the enzyme membrane into powder led to cancellation of the effect. Using coupled reaction-diffusion models, Goldman and others showed that substrate and product concentration gradients are established within an enzyme membrane owing to diffusional limitations on the translocation of substrate and product. Hence substrate depletion is reflected in an increase in the value of the experimentally determined Michaelis constant. Moreover, the full enzymic activity of the membrane could be realized only in the case of very poor substrate, viz., extremely slow reactions (Goldman *et al.*, 1968a,b; Sundaram *et al.*, 1970; Selegny *et al.*, 1971). These studies introduced the concept of a microenvironment generated by an enzymic reaction taking place in a sterically constrained system.

Extension of the experimental investigation of enzyme membranes to very fast enzymes, such as alkaline phosphatase or glucose oxidase (Goldman *et al.*, 1971a,b; Broun *et al.*, 1969; Selegny *et al.*, 1971), showed that theoretical models based solely on internal diffusional resistances within a porous support could not fully account for the highly perturbed values of the Michaelis constants. The experimental findings could, however, be explained if in addition the existence of

concentration gradients across unstirred layers (the Nernst diffusion layers; Nernst, 1904) around the enzyme membranes were assumed (Goldman *et al.*, 1971a,b; Goldman, 1973).

In parallel, intensive work was being carried out on continuous-flow packed-bed and continuous-stirred tank-enzyme reactors; analytical expressions correlating the degree of conversion of substrate for systems obeying Michaelis–Menten kinetics with the rate of flow of solution through the column or the agitation rate in the case of stirred-tank reactors were developed (Lilly *et al.*, 1966, 1974; Lilly and Sharp, 1968; Lilly and Dunnill, 1972; Hornby *et al.*, 1968). Here again, the high values obtained for the Michaelis constants, which could not be accounted for by the simple kinetic models, led to the assumption that substrate concentration gradients across a stagnant, unstirred layer surrounding the immobilized enzyme particles were responsible for the anomalous kinetic behavior and hence to the extension of the theoretical models to include these effects (Lilly and Sharp, 1968; Hornby *et al.*, 1968).

The kinetic consequences of diffusional limitations in immobilized enzyme systems were further demonstrated in several experiments: Axén showed that, in the case of particulate chymotrypsin–Sepharose conjugates of highly perturbed Michaelis constants, the values of the latter dropped to essentially those of the native enzyme, after solubilization with dextranase (Axén *et al.*, 1970). Mosbach, who investigated the behavior of multienzyme systems, showed that, in the case of two enzymes that carry out consecutive reactions, the initial rate of appearance of the last product is enhanced when the enzymes are immobilized together; moreover, the lag usually observed in the appearance of the last product, with the soluble enzymes, was absent with the immobilized two-enzyme system. The observations suggested that owing to the spatial proximity of the two enzymes on the supporting matrix, and the diffusional resistances deriving from unstirred layers, higher local concentrations of the intermediate product could be attained in the immobilized two-enzyme system (Mosbach and Mattiasson, 1970; Mattiasson and Mosbach, 1971; Gestrelius *et al.*, 1972, 1973; Mosbach *et al.*, 1974a,b). A theoretical analysis based on these assumptions (Goldman and Katchalski, 1971) gave predictions in good agreement with the experimental observations.

The microenvironment and diffusional resistance concepts roughly outlined here in the sequence of their formulation have been applied to rather sophisticated model enzyme membranes and particulate immobilized-enzyme systems to study aspects of structure-modulated kinetics; these include the precise physical meaning of experimentally

determined kinetic parameters (Engasser and Horvath, 1973, 1974a; Hamilton *et al.*, 1974a,b; Moo-Young and Kobayashi, 1972; Kobayashi and Laidler, 1973; Buchholz and Rüth, 1976), regulatory effects, ion-selective, facilitated and active transport (Mitz, 1971; Broun *et al.*, 1970, 1972; Selegny *et al.*, 1971; Lecoq *et al.*, 1975; Goldstein, 1972, 1973; Gestrelius *et al.*, 1972, 1973; Kasche and Bergwall, 1974; Johansson and Mosbach, 1974a,b; Engasser and Horvath, 1974b; Thomas *et al.*, 1974; Thomas and Broun, 1973; Hervagault *et al.*, 1975), as well as new concepts, such as asymmetrical behavior, hysteresis, and oscillations (Thomas *et al.*, 1972; Caplan *et al.*, 1973; Naparstek *et al.*, 1973, 1974; Thomas and Caplan, 1976). Moreover, serious attempts are being made to apply the experience accumulated in the study of model systems for the quantitative description of metabolic pathways and metabolic compartmentalization in intact cells (Blum and Jenden, 1957; Roughton, 1959; Connett and Blum, 1971, 1972; Connett *et al.*, 1972; Raugi *et al.*, 1973a,b, 1975; Liang *et al.*, 1973; Blum, 1974; Srere *et al.*, 1973; Srere and Mosbach, 1974). Most of these aspects are discussed in depth in the chapter by Engasser and Horvath in this volume.

The study of the engineering aspects of coupled mass transfer-reaction kinetics, initiated in the early work on enzyme columns (Lilly *et al.*, 1966; Lilly and Sharp, 1968), led through integration of the approaches of the physical chemist dealing with the fundamentals of diffusion and the chemical engineer well versed in mass-transfer and heterogeneous catalysis, to a high degree of sophistication in enzyme-reactor analysis and design (Wingard, 1972a,b; O'Neill, 1972; Lilly and Dunnill, 1972; Lilly *et al.*, 1972, 1974; Vieth and Venkatasubramanian, 1974). The reader is referred to the chapter by Vieth *et al.* in this volume for a comprehensive survey of current status of design and analysis of immobilized-enzyme flow reactors.

The advances in the study of the basic properties of immobilized enzymes were accompanied by venues into laboratory-scale application and in analysis.

The controlled degradation of complex biological macromolecules with immobilized enzymes was first described by Cebra and coworkers, who isolated F_{ab} and F_c fragments from short digests of rabbit γ-globulin with immobilized papain (Cebra *et al.*, 1961, 1962; Cebra, 1964). Along the same lines, Lowey and others used immobilized derivatives of trypsin and papain to obtain and characterize subfragments of myosin in their studies on the structure of muscle proteins (Lowey *et al.*, 1966, 1967, 1968; Slayter and Lowey, 1967; Wolodko and Kay, 1975). Similar work on other biological macromolecules has