Immobilized Enzymes

An Introduction and Applications in Biotechnology

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Abbreviations and Symbols

A	Surface area
D	Diffusion coefficient
d	Pathlength of diffusion
E_T	Total enzyme concentration
H_e^+	Hydrogen ion concentration in bulk phase
H_i^+	Hydrogen ion concentration in enzyme phase (microenvironment)
h_s	Substrate transport coefficient
J_{H^+}	Rate of flux of hydrogen ions
K'	1st order rate constant
K_v	Half-saturation constant (substrate concentration at $V_s/2$)
p	Partition coefficient
P	Product concentration
pH_e	pH of the bulk phase
pH_i	pH of the enzyme phase (microenvironment)
S	Substrate concentration
S_o	Substrate concentration in bulk phase
S_i	Substrate concentration in enzyme phase (microenvironment)
V	Reaction velocity
V'_{max}	Saturation velocity at pH optimum
V_s	Saturation velocity for an immobilized enzyme
V(diff)	Diffusion velocity of substrate
Δ	Ionic partition coefficient

Preface

The immobilization of enzymes by fixing them in some way on to an inert and usually insoluble polymer matrix, is an area of research that is currently generating much interest among biochemists, organic and physical chemists, microbiologists, biomathematicians, biophysicists, and chemical engineers. It is probably because of this multidisciplinary approach that most of the literature on the subject is written by and for the active research worker and is largely inaccessible to the 'non-expert'. This book is an attempt to fill that gap and provide an introductory text to this fascinating subject; it is not intended as a reference book, sufficient of these exist. Unnecessary and confusing detail is thus omitted and explanation of many of the effects of immobilization is kept as unmathematical as is humanly possible. Understanding, therefore, is based on the reader gaining a thorough intuitive grasp of the concepts involved.

Research on immobilized enzymes has been carried out for the past two decades from two principal standpoints. First it was apparent that there was vast potential for using immobilized enzymes as recoverable, stable, and specific industrial catalysts. A great deal of research has thus gone into the immobilization of enzymes of commercial importance and the associated problems of reactor design and scale-up procedures. However, despite the quantity and quality of the research in this field the potential has as yet been little realized.

Second, immobilized enzymes have been studied with a view to discover some of the effects a heterogeneous environment may have on an enzyme-catalysed reaction. Study of highly purified enzymes in dilute solution under Michaelis–Menten conditions reveals a great deal about the way in which enzymes will behave under such conditions. Unfortunately for the enzymologist, most intracellular enzymes are neither working under Michaelis–Menten conditions nor are they in dilute solution, but in a complex heterogeneous environment. Thus for a better understanding of the behaviour and control of enzymes functioning *in vivo*, many attempts have been made to study the effect of immobilization and a defined heterogeneous environment on the enzyme and then relate this to the *in vivo* situation.

This book is divided into five chapters. Chapter 1 covers briefly some of the methods by which enzymes may be immobilized and the factors that lead to

the choice of a particular method. Chapter 5 details some easy methods for preparing immobilized enzymes. Chapter 2 relates some of the effects that immobilization may have on the kinetic parameters of an enzyme-catalysed reaction. Chapter 3 explores some of the possibilities and problems in the commercial and medical applications of immobilized enzymes. In Chapter 4 an attempt will be made to illustrate the way in which immobilized enzyme systems can be made to mimic biological systems. The information obtained from such model systems will be discussed with particular reference to its relevance to our understanding of the cell and its biochemistry.

I would like to acknowledge the help and cooperation of all my colleagues during the period of the preparation of this book. In particular my thanks to Elaine Johnson and Vyvyan Ellen for their help in the preparation of this manuscript. I would also like to thank my wife, Marilyn, for her understanding, enthusiasm, encouragement and endless cups of late night coffee without which I doubt that this project would have come to fruition.

Contents

Chapter 1

Techniques of Immobilization

I. IMMOBILIZATION — THE PRINCIPLES AND THE PROCESS

Enzyme immobilization may be defined as the imprisonment of an enzyme molecule in a distinct phase that allows exchange with, but is separated from, the bulk phase in which substrate effector or inhibitor molecules are dispersed and monitored. The enzyme phase (see Figure 1) is usually insoluble in water and is often a high molecular weight, hydrophilic polymer (e.g. cellulose). The imprisonment of the enzyme may be achieved by various means. The enzyme can be covalently bonded to, adsorbed on to, or physically entrapped within the enzyme phase.

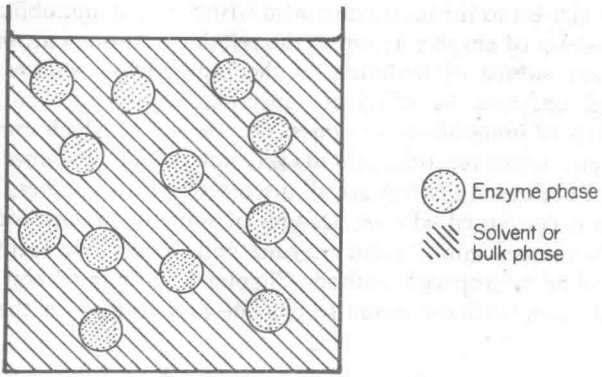


Figure 1. Definition of enzyme and bulk phases in immobilized enzyme system

The use of the term 'immobilization' can lead to confusion, for it implies, incorrectly, that the enzyme molecule can never move about within its distinct phase. Immobilization is, however, a more apt term for the process than the once much used term 'insolubilization' as water-soluble immobile enzymes can be prepared. The reader must, however, beware as both terms are used interchangeably in the literature.

The nature of the immobilized enzyme preparation will obviously depend upon the character of the enzyme phase. For example, the most common form of immobilized enzyme is that in which the enzyme molecule has been covalently bonded on to an insoluble polymer such as cellulose or polyacrylamide.

The polymer, however, may be in the form of a particulate powder or in sheet form as a membrane. The enzyme may even be covalently bonded on to itself (or another inert protein), forming an insoluble, but active, polymeric enzyme. Yet another approach is to attach the enzyme to a polymer using electrostatic or other non-covalent bonding mechanisms. Alternatively the enzyme need not be bound to the polymer, but may be trapped within it, the polymer forming a net-like matrix around the enzyme, the pores in the net being too small to allow the escape of the enzyme, but large enough to permit the entry of low molecular weight substrates. A variation of this last technique lies in the use of phospholipid bilayers as the enzyme phase. In this case the enzyme may either be in aqueous solution surrounded by a phospholipid barrier (see liposomes p.64) or actually be 'dissolved' in the hydrophobic portion of the bilayer (see p.117). Figure 2 depicts diagrammatically these various forms of immobilized enzyme.

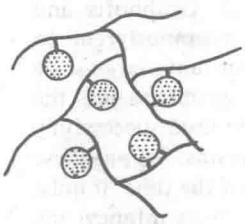
Immobilization often causes a dramatic change in the apparent measured parameters of the enzyme-catalysed reaction. For example, the maximum velocity of reaction, Michaelis–Menten constant, temperature optimum, pH optimum, effect of inhibitors may all be changed when an enzyme is immobilized; the degree and nature of this change will depend not only on the immobilization method used, but also on the enzyme reaction. These effects are of great interest to the biochemist who wishes to use immobilized enzymes as model systems of enzyme action in the cellular environment, but they may be a constant source of irritation to the industrialist who wishes to use immobilized enzymes as efficient, specific, recoverable catalysts. The industrial uses of immobilized enzymes, the manner in which immobilization may affect an enzyme reaction, and the way immobilized enzymes can be used as models of biological systems are all discussed in later chapters. The rest of this chapter is concerned with an account of some of the methods that have been used to prepare immobilized enzymes and, finally, the factors affecting the choice of an appropriate method. Chapter 5 outlines several methods of immobilization in sufficient detail to provide easy recipes for the uninitiated to try.

II. COVALENT BONDING TO ACTIVATED POLYMERS

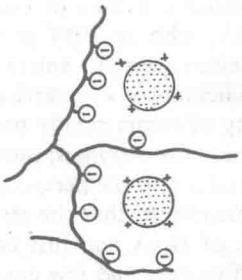
Immobilization by covalent bonding to activated polymers is probably the most extensively used method, for although often tedious it provides an immobile enzyme that is firmly bound to its polymeric support. The range of polymers and chemical coupling procedures used is enormous and so the descriptions here cover only the most important methods developed over the years.

1. The Emergence of the Technique

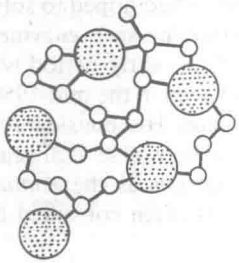
The history of enzyme immobilization goes back to the late 1940s. In 1949



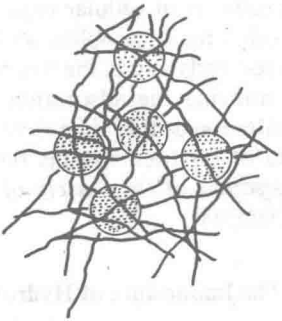
(1) Covalent bonding



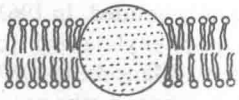
(2) Electrostatic bonding



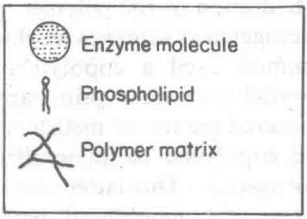
(3) Copolymerization

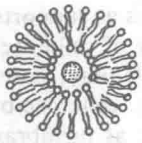


(4) Polymer entrapment

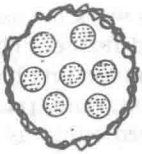


(5) Hydrophobic interaction





(6) Liposmal entrapment



(7) Encapsulation

Figure 2. Possible modes of enzyme immobilization

Michael and Ewers used the azide derivative of carboxymethylcellulose to immobilize a variety of proteins. However, it was not until Grubhofer and Schleith, who in 1954 used a diazo derivative of poly-*p*-aminostyrene to immobilize pepsin, amylase, and carboxypeptidase, that any large-scale experimentation was carried out in this field. One of the problems was the scarcity of commercially prepared polymers which could be used successfully to immobilize enzymes; most were synthetic hydrophobic resins. To put these discoveries into the perspective of biochemical knowledge of the time, it must be remembered that the structure of proteins was a field in its infancy, the nature of DNA had just been discovered, biochemical genetics had not yet been invented, and few convincing hypotheses for the structure of biological membranes had been advanced. The discovery that the nature of the polymeric support used influenced the apparent kinetics of the immobilized enzyme must have been not a little disquieting to those who had hoped to solve the problems of cellular organization and control by studying such enzymes. The other major problem was that a lot of the work of this early period was both too early and in the wrong place. For example, McLaren in the mid-1950s was studying the adsorption of enzymes on to kaolinite. His considerable contribution at that time went largely unnoticed, because he was several years ahead of his time and his reports appeared in such journals as the *Annual Proceedings of the Society of Soil Science*, a journal not often consulted by biochemists!

2. The Importance of Hydrophilicity

It was not until the work of Manecke in 1961 and his observation that, in general, the level of activity of an immobilized enzyme depends on the degree of hydration of the polymer matrix, that polymers other than cellulose were investigated as supports to which enzymes could be covalently bonded. In 1963 Rimmon used a copolymer of L-leucine-*p*-amino-D,L-phenylalanine to immobilize chymotrypsin, papain, pepsin, and streptokinase. Manecke in 1962 pioneered the use of methacrylic acid-3-fluoro-4,6-dinitroanilide: methacrylic acid copolymer to immobilize pepsin, amylase, invertase, and alcohol dehydrogenase. This latter enzyme was the first oxido–reductase enzyme to be successfully immobilized, until that time all other immobilized enzymes were hydrolases.

Concurrently with this work the use of cellulose derivatives as supports for enzymes was investigated further. The continued popularity of cellulose was due to its inherent advantages; its high hydrophilicity, availability, potential for varied derivatization, and relative ease with which cellulose-based polymers can be produced either as particulate powders or as membranous films. Thus Mitz and Summaria in 1961 reported the coupling of trypsin and chymotrypsin to diazotized *p*-aminobenzoyl cellulose and also to the hydrazide derivative of carboxymethylcellulose. These two methods are still in use today.

3. Bridging the Gap

It is perhaps more useful when using cellulose as a polymer support not to have to build the reactive group into the cellulose (as in *p*-aminobenzoyl cellulose), but to use a chemical 'bridge' between the cellulose and the enzyme molecule. The requirement for this bridge molecule is that it must be small and, once reacted with the cellulose, have a group capable of reacting with the enzyme. Such a compound is cyanuric chloride (trichlorotriazine). This has three reactive C—Cl bonds (see Figure 3). One is first reacted with the cellulose (very rapidly), the second with the enzyme, and the third may be reacted with any convenient compound. Using this method Kay and co-workers (Kay *et al.*, 1968; Kay and Crook, 1967) linked galactosidase, lactate dehydrogenase, pyruvate kinase, and creatine kinase to cellulose in the form of filter paper. The particular advantage of cyanuric chloride as a bridge molecule is that the ionic nature of the enzyme–cellulose complex depends upon the ionic charge of the bridge molecule. This can be neutral, cationic, or anionic depending upon the nature of the compound added to the third C—Cl bond. Thus enzyme–cellulose complexes can be made polycationic by this method, a great advantage since most other methods tend to result in polyanionic complexes.

Another useful bridge molecule is glutaraldehyde. This contains two aldehyde groups at either end of a $(CH_2)_3$ unit. The aldehyde groups will react at neutral pH values with free amino groups. Thus one end of the glutaraldehyde molecule may be attached to the support, the other to the enzyme.

The most common activation method in use today is that involving cyanogen bromide (CNBr) (Axen *et al.*, 1967; Porath, 1974). The exact way in which this molecule reacts with cellulose has yet to be ascertained, but at high pH values it apparently reacts readily with the hydroxy groups of polysaccharides and the derivative will then react with free amino groups on the enzyme in mildly alkaline solutions. Problems are nevertheless experienced with this method (apart from the handling of cyanongen bromide) and it is becoming apparent that the bonding, particularly of small molecules, is not altogether stable.

4. Disadvantages of and Alternatives to Cellulose

Polysaccharides are not the ideal support material for enzyme immobilization as they suffer from two serious drawbacks. First, polysaccharides are susceptible to microbial attack. Nothing is more infuriating than finding a microbial growth devouring a precious immobilized enzyme preparation, particularly in a large-scale industrial application! Second, cellulose shows a high degree of non-specific adsorption of protein. Consequently, at the end of the preparative procedure the immobilized enzyme must be washed extensively in buffers of high ionic strength. This process may not only be expensive on a large scale but may often inactivate the enzyme, especially if it is dimeric or polymeric in its active form (see p.17).

Much work has thus been devoted to the search for polymer support materials that are hydrophilic but microbially non-degradable. Levin and Goldstein independantly reported, in 1964, the use of ethylene–maleic anhydride as a support for various enzymes. Other materials which have been successfully employed include glass (Weetal, 1969), and nylon (Inman and Hornby, 1972). A more general approach has arisen from the work of Inman and Dintzis who in 1969 pioneered the use of various derivatives of polyacrylamide. Many varieties of ready prepared acryl copolymers are now commercially available, the reactive groups of which commonly include diazo, aldehyde, carboxylmethyl, and hydrocyanate derivatives. A useful member of this group of polymers is a soluble acryl polymer derivative suitable for preparing soluble immobile enzymes.

III. COPOLYMERIZATION WITH MULTIFUNCTIONAL REAGENTS

Multifunctional reagents can be used not only to link enzyme molecules to cellulose or other polymers, but also to link enzyme molecules to each other. Although such a matrix may contain just enzyme molecules, it is usually in the interests of economy to copolymerize the enzyme with an inert protein such as albumin in order to increase the bulk of the final product.

The most commonly used multifunctional reagent is glutaraldehyde. Aldehydes in general and glutaraldehyde in particular have long been used by histologists as fixing agents; Baker in 1910 noted the protein gelling action of aldehydes. In fact, despite its age, Baker's observation is pertinent today, for it is extremely difficult to precipitate an enzyme matrix out of solution using glutaraldehyde, at best the solution usually gels. In order to produce an insoluble matrix of enzyme and glutaraldehyde it is necessary either to cause the glutaraldehyde to polymerize or to precipitate the enzyme (or adsorb it on to some insoluble surface). The net effect is either to increase the length of the bridging molecule or to decrease the intermolecular distance of the enzyme molecules. Using such approaches Richards (1964), Ogata (1968), Jansen (1969), and Habeeb (1967) have cross-linked and insolubilized carboxypeptidase A crystals, subtilisin novo, papain, and trypsin respectively.

Membranous sheets of cross-linked enzyme of controlled pore size can be prepared with glutaraldehyde. The enzyme is adsorbed on to a preformed cellulose nitrate membrane and then glutaraldehyde introduced to cross-link the enzyme molecules in place around the cellulose nitrate fibres. The cellulose nitrate can then be dissolved away with methanol leaving an enzyme membrane (Goldman *et al.*, 1968b).

Although many other multifunctional reagents have been used to cross-link enzymes, for example *N,N'*-bisdiazobenzidine-2,2'-disulphonic acid or 2,4-dinitro-3,5-difluorobenzene, only glutaraldehyde has found extensive use. This is probably because it reacts with proteins readily under mild conditions — 2,4-dinitro-3,5-difluorobenzene requires highly alkaline solutions to react and the presence of organic solvent to dissolve it — and is probably the least

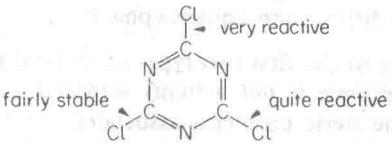
toxic of the bifunctional reagents used; *N,N'*-bisdiazobenzidine-2,2'-disulphonic acid is a potentially explosive carcinogen!

The major problem with this type of method is that the bifunctional reagent will often preferentially attack the active site of the enzyme, thus rendering it inactive. However, when it can be made to work, for example by reversibly blocking the active site, it works well.

IV PHYSICAL ADSORPTION

The oldest method of enzyme immobilization is that of physically adsorbing the enzyme on to a polymer matrix without covalent bonding. It is exceedingly easy to perform, the adsorbent and enzyme are stirred together for some time, but yields (enzyme bound per unit of adsorbent) are low and the enzyme is often partially or totally inactivated. A variety of adsorbents have been used; the binding forces may be ionic, hydrophobic, hydrogen bonds, or Van der Waals' interactions.

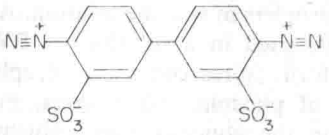
Unfortunately there is an inherent snag in the use of physical adsorption for enzyme immobilization. It is embodied in the principle 'what goes up must come down', that is the reversible nature of the bonding of enzyme to support may lead to desorption of the enzyme at a critical time. One factor which often causes desorption is the addition of substrate to the enzyme preparation. This is a particular hazard, for although other factors which might cause desorption (such as fluctuations in pH, temperature, or ionic strength) can be controlled,



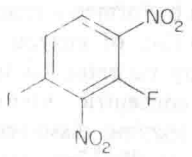
Cyanuric chloride

$OHC{\cdot}CH_2{\cdot}CH_2{\cdot}CH_2{\cdot}CHO$

Glutaraldehyde



NN'-Bisdiazobenzidine-2,2'-Disulphonic acid



2,4-Dinitro-3,5-Difluorobenzene

Figure 3. Multifunctional reagents

no enzyme can work without its substrate. It is interesting that despite this limitation it was just such a method that was used in the first commercial application of an immobilized enzyme.

The high adsorption of proteins by cellulose, which is a problem in the use of cellulose for the covalent bonding of enzymes, is here a great advantage. Cellulose based ion-exchange resins (e.g. carboxymethylcellulose and DEAE cellulose) have been extensively used and high adsorbent capacities have been demonstrated (up to 15% w/w protein : cellulose). Other materials often used, but with markedly inferior capacities, include polystyrene resins, kaolinite, collagen, alumina, silica gel, and glass.

One interesting development of the technique of immobilization by adsorption is in the use of an effector or activator of an enzyme, itself attached to a water-insoluble polymer, to bind that enzyme. For example, Fukui *et al.* (1975) demonstrated the immobilization of tyrosinase and tryptophanase by adsorption on to an insoluble derivative of pyridoxal-5′phosphate, an activator of these enzymes. This method has the added advantage that the enzyme is not only specifically adsorbed on to the polymer, but is also activated by the same process.

V ENTRAPMENT AND OCCLUSION

The entrapment of an enzyme molecule can be achieved in one of three ways.

1. Inclusion within the matrix of a highly cross-linked polymer.
2. Separation from the bulk phase by a semipermeable 'microcapsule'.
3. Dissolution in a distinct non-aqueous phase.

An important feature of the first two types of method in this group (1 and 2 above) is that the enzyme is not actually attached to anything. There are therefore none of the steric problems associated with covalently or electrostatically binding an enzyme on to a polymer, for example binding the enzyme in such a way that its active site is obstructed by a portion of the polymer matrix (see p.21). In general, entrapment methods are performed by dissolving the enzyme in a solution of the chemicals required for synthesis of the enzyme phase and then treating this solution so that a distinct phase is formed. Cross-linked polyacrylamide gels may be formed by dissolving enzyme, acrylamide and methylene-bis-acrylamide in buffer and initiating polymerisation. The gel formed is mechanically disrupted to form small, enzyme loaded particles (Figure 4). Semipermeable microcapsules of nylon, of defined size and porosity, can be formed around droplets of enzyme solution. Alternatively, an aqueous solution of enzyme dispersed in a solution of PVC or cellulose triacetate may be extruded to form fibres containing droplets of enzyme. Liposomes, concentric spheres of phospholipid bilayers, may be used to encapsulate enzyme dissolved in the aqueous compartments between the bilayers. The medical importance of such preparations will be discussed later (p.64). Phospholipid bilayers have also been used as a lipid membrane in which the enzyme isomaltase/sucrase is 'dissolved' (Storelli, *et al.* 1972). This