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Developments in Immobilized-Enzyme Technology

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General introduction

Enzyme immobilization can be broadly described as a process by which an enzyme is converted from a homogeneous catalyst to a heterogeneous catalyst. It has been defined by Trevan (1980) as a process by which an enzyme is separated into a distinct phase (usually water-insoluble and often of high molecular weight) which is separate from the bulk substrate-containing phase. In the enzyme reaction, exchange of substrate and product between the two phases occurs by diffusion. Chibata (1978) used a similar concept when he defined immobilized enzymes as being physically confined or localized in a defined region. In addition, he included in his definition two valuable properties associated with them, namely reusability and the ability to be used for continuous processes. These definitions encompass a wide variety of immobilization techniques, ranging from relatively simple methods such as precipitation of enzymes by reaction with bifunctional reagents such as glutaraldehyde, to the more complex multistage processes involved in some of the chemical reaction processes used to bind enzymes to polymer supports. The major impetus behind the development of enzyme immobilization techniques has been that they convert enzymes into versatile tools capable of use for a wide variety of applications. For example, they can be used for carrying out industrial processes, such as the resolution of amino acids. They are being used for industrial and clinical analysis and have found application in medicine for therapeutic purposes.

Immobilized enzymes are not new. Many of the 2000 listed enzymes either act intracellularly where they may be associated with intracellular particles or membranes or, if they are extracellular enzymes, they may act in the presence of particles, colloids or gels. Indeed, the development of immobilization systems has allowed investigations into the action of enzymes under conditions more akin to their native environment (McLaren and Packer, 1970). One of the first reports of an artificially prepared immobilized enzyme was that of Nelson

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and Griffin (1916) who adsorbed invertase to charcoal with no apparent loss of activity. At that time the potential applications of immobilized enzymes were not realized and a gap of almost forty years elapsed before the publication of Grubhofer and Schleith (1953) describing the covalent attachment of α-amylase, pepsin, ribonuclease and carboxypeptidase to a diazotized derivative of polyaminopolystyrene. The intervening period saw the development of immunoadsorbents — insolubilized antigens and antibodies — developed to enable the isolation of the corresponding antibody or antigen. An important prerequisite in this work was the achievement of good loadings on to the support with minimal inactivation of binding sites. If binding sites are replaced by active sites, then these requirements hold for enzyme immobilization, but it was not until the latter half of the 1960s that interest in enzyme immobilization increased significantly. This is graphically illustrated by Chibata (1978) in a plot of the number of papers and patents published up to 1973. Over the last decade a large number of publications — papers, patents, books — have appeared and immobilized enzymes have now become part of the new biotechnology.

In the early period of immobilized enzyme development, attention was focused primarily on immobilization methodology. However, as the application of immobilized enzymes developed it became apparent that immobilization systems tailored to meet the requirements for each application were needed and a more integrated style of development resulted. In this review, immobilization methodology will be discussed, as well as the various uses for which such enzymes are intended and the constraints that are attendant in the diverse fields of application. The intention is not to provide an extensive bibliography of the methods, for many excellent reviews are already available containing this information, but to provide an overview of the pros and cons of the different methods with respect to the areas of application. An indication will be given of the new areas of development. Of particular interest is the work of Klibanov and co-workers at the Massachusetts Institute of Technology who, realizing that enzyme availability is as much a constraint to their use as the availability of suitable immobilization techniques, are investigating new reactions for commercially available enzymes. Finally, attention will be given to the alteration of enzyme properties which are caused by the immobilization processes and which result from the conversion of a homogeneous catalyst (soluble enzyme) to a heterogeneous catalyst (immobilized enzyme) with all the attendant micro-environmental and diffusional limitation effects.

While the bulk of this review relates to immobilized enzymes, reference must be made to the rapidly growing field of cell immobilization. The use of immobilized cells has become of increasing importance where multienzyme processes are being considered and where enzymes requiring cofactors are being used, because such cells obviate the need to purify and immobilize a number of enzymes and they contain cofactor-regenerating systems. It is possible to reactivate an immobilized live-cell system by incubation in a nutrient medium for a short period. However, the specific activity of immobilized cells is usually lower than that of immobilized enzymes, and unwanted side-reactions may occur due to the presence of enzymes other than those needed for the required reaction. Nevertheless, commercial immobilized cell processes have been developed, e.g. organic acid production, and the

potential value of immobilized cells is generally recognized. This field has been extensively reviewed by Cheetham (1980) (*see also* Chapter 11 of this volume).

Enzyme immobilization methodology

INTRODUCTION

Before discussing the wide variety of techniques that have been developed for immobilizing enzymes, it is pertinent to refer to attempts that are being made to standardize the nomenclature of immobilized enzymes and to introduce some conformity in their characterization in order to facilitate easier and more valid comparisons of different immobilized-enzyme systems. At the 1973 Enzyme Engineering Conference an *ad hoc* committee recommended the use of the term 'immobilized enzyme' to describe all preparations where an enzyme is associated with a supporting polymer (Sundaram and Kendall Pye, 1973). It was envisaged that this would replace the plethora of alternatives such as 'water-insoluble enzyme', 'fixed enzyme', 'matrix-supported enzyme', that had appeared in the literature. The committee also recommended that immobilized enzymes should be classified under two main headings: 'bound' and 'entrapped'. Bound enzymes were subdivided into two smaller groups: 'adsorbed' and 'covalently bound', and entrapped enzymes were similarly divided into two groups: 'matrix entrapped' and 'microencapsulated'. This classification will be used when discussing immobilization methodology. The committee also recommended that all enzyme activities should be initial rates, measured under defined conditions and expressed as μkatals per mg of dry preparation or per unit area in the case of surface (e.g. tube, plate, membrane) bound enzymes. Whenever possible, data was requested on the drying conditions for, and the protein content of, the bound enzyme as well as the specific activity of the free enzyme. Because of the influence of the support and the binding process on the kinetics of immobilized enzymes, it was recommended that all kinetic constants have the suffix (app), e.g. $K_{m\,(app)}$, $V_{max\,(app)}$. Where stability data is presented, precise conditions under which it is measured were requested. Where covalent coupling systems are developed, it was recommended that data should include the number of reactive groups per unit weight of support and the maximum loading achievable for a small molecule as well as the larger enzyme molecule. The problems caused by variable characterization of immobilized enzymes and constructive recommendations for avoiding these difficulties form the subject matter of a Dechema monograph (Buchholz, 1979).

Several excellent reviews of enzyme-immobilization techniques have been published (Zaborsky, 1973; Goldstein and Manecke, 1976; Mosbach, 1976; Chibata, 1978) and a compilation of references to recent papers and patents is published in the journal *Enzyme and Microbial Technology*.

BINDING TECHNIQUES

Adsorption processes

One of the simplest procedures for immobilizing an enzyme is to contact it with a support under conditions in which physical bonding can occur. The process

can be carried out by contacting enzyme and support in a stirred reactor or by percolating the enzyme through a packed bed, tube or membrane formed from the support material. Nozawa *et al.* (1982) even used dry milling to adsorb trypsin to chitin and chitosan.

Apart from operational simplicity, adsorption procedures have the advantage that they can be applied to a wide range of organic and inorganic supports. Examples have included ion-exchange resins, celluloses, Sephadexes, agaroses, collagen, starch, gluten, brick dust, silica, carbon, alumina, titania, bentonite, glass, ceramics and metal supports (Zaborsky, 1973; Goldstein and Manecke, 1976; Chibata, 1978). Supports can be modified to improve adsorption by the attachment of cofactors such as pyridoxal phosphate (Fukui *et al.*, 1975) or by the use of hydrophic side chains. Alkylated derivatives of Sepharose were used by Hofstee and Otillio (1973), Visser and Strating (1975) and Tramper *et al.* (1979). Tritylated agarose was used by Cashion *et al.* (1982).

An advantage of adsorption processes compared with covalent attachment methods is the minimum distortion of the enzyme necessary to achieve binding and hence the potential for higher retention of enzyme activity. A further advantage is that enzyme can be removed from the support relatively easily since no disruption of chemical bonds is needed. Hence reuse of the support by readsorbing enzyme to it is feasible. The economic advantages of being able to reuse the support was an important criterion in the decision to use an adsorbed aminoacylase for large-scale amino-acid resolution (Tosa *et al.*, 1969; Chibata, 1978).

However, this property can also be a disadvantage because the inherent problem associated with adsorbed enzymes relates to the weakness of physical binding forces such as ionic links, hydrophobic interactions, hydrogen bonding and Van der Waal's forces and the relative ease with which they can be broken by changes in pH, temperature or ionic strength with concomitant desorption of the enzyme. Loss of enzyme reduces enzyme reusability and results in enzyme being present in the final product. This may well explain the relatively small number of publications in which adsorbed enzymes have been described, compared with those in which covalent binding methods have been used (of a random selection of 56 papers published in 1982 only seven related to adsorption processes compared with 43 in which covalent attachment was used). However, it is salutary to be reminded that the first industrial process using an immobilized enzyme used an aminoacylase adsorbed to DEAE-Sephadex for continuous amino-acid resolution. In this process a solution of *N*-acetyl-DL-amino acid is passed through the immobilized enzyme in a column reactor, the acetyl group being cleaved from the *N*-acetyl-L-amino acid. The resultant L-amino acid is then extracted, and the *N*-acetyl-D-amino acid is racemized and returned to the reactor. The development of this process is described in detail by Chibata (1978). It is interesting to note that a batch process using soluble aminoacylase had been operating since 1953, but the immobilized enzyme process had the advantages of cheaper labour costs, lower enzyme cost, more efficient extraction of product (since enzyme removal procedures were not needed) and could be used to run a continuous process. Several immobilization procedures (physical binding, covalent binding, entrapment and microencapsulation) were investigated, of which three —

adsorption to DE-Sephadex, covalent binding to iodoacetyl cellulose and entrapment in polyacrylamide gel — looked promising. Of these, the enzyme adsorbed to DE-Sephadex was chosen because of its ease of preparation, good activity and stability, and its ability to be regenerated easily.

The first large-scale immobilized enzyme process for the production of high-fructose corn syrups, developed by Clinton Corn Products, utilized glucose isomerase adsorbed to DEAE-cellulose. The process involved the liquefaction and saccharification of corn starch followed by the isomerization process to produce fructose. As with the amino-acid resolution process, a continuous immobilized-enzyme system replaced an existing batch process using soluble enzyme. The immobilized enzyme is used in multiple shallow-bed reactors and its half life is reported to be several hundred hours (Thomson, Johnson and Lloyd, 1974; Mermelstein, 1975; *see also* Poulsen (1984) in Volume 1 of this Series).

A process for the manufacture of 6-aminopenicillanic acid by the dephenylacetylation of benzyl penicillin G using a penicillin acylase from *Bacillus megaterium* adsorbed to bentonite has also been reported (Heuser, Chiang and Anderson, 1969).

A variation of the adsorption process is to follow it with a treatment with a bifunctional reagent such as glutaraldehyde to 'staple' the enzyme in place. Haynes and Walsh (1969, 1974) used this technique to immobilize enzymes on to colloidal silica. This procedure uses two immobilization procedures — adsorption and cross-linking — both of which have inherent disadvantages. The disadvantage of the adsorption process has already been discussed. Cross-linking of enzymes results in an insoluble polymer which has a poor physical form generally unsuited for widespread application. In combining the two processes it is possible to produce a physically acceptable enzyme preparation with improved stability. The process can be applied to enzymes adsorbed to membranes as well as to particulate solids. Several cross-linking agents have been reported but only two — bisdiazobenzidine-2,2-disulphonic acid and glutaraldehyde — appear to have had widespread use (Goldstein and Manecke, 1976). Glutaraldehyde is particularly useful for large-scale use because of its ready availability in large amounts and its cheapness. Savidge and Powell (1977) have described the adsorption and cross-linking of penicillin acylase to polymethacrylate polymers.

Although adsorption as an immobilization procedure has not generally been regarded very favourably, the examples cited above demonstrate that it is a useful technique, particularly because of its ease of application. It is obviously best suited to enzyme-support systems for which the optimum pH of adsorption is near the pH at which the enzyme will be used. The addition of a cross-linking procedure can assist in the stabilization of the enzyme–polymer conjugate.

Although adsorption techniques are attractive in their simplicity, much more attention has been focused on developing covalent attachment techniques.

Covalent coupling processes

Enzymes, by virtue of being composed of chains of amino acids, have a number of reactive side chains which can be utilized for forming chemical bonds with

appropriately activated supports. The functional groups present in protein molecules are amino groups (α and ε), carboxyl groups (α, β or γ), sulphydryl groups, hydroxyl groups, imidazole groups and phenol groups. The range of coupling processes is limited only by the ingenuity of the organic chemist. Both organic and inorganic supports have been used and many extensive reviews of the methodology are available (Zaborsky, 1973; Goldstein and Manecke, 1976; Mosbach, 1976; Chibata, 1978; Kent, Rosevear and Thomson, 1978).

One of the most commonly used covalent attachment techniques is the cyanogen bromide (CNBr) activation of Sephadex and Sepharose first reported by Axen, Porath and Ernback (1967) and Porath, Axen and Ernback (1967). The popularity of the technique was reported by Zaborsky (1973), Goldstein and Manecke (1976) and Chibata (1978), and was noted by the present author in the course of preparing this review. Commercial preparations of CNBr-activated Sepharose are available and enzyme immobilization is simple to carry out and requires only mild conditions. The cyanogen bromide reacts initially with the hydroxyl groups of the polymer to produce a cyanate ester (O−C ≡ N). Where vicinal hydroxyl groups are present, as in dextran, cellulose and Sephadex, a rapid rearrangement occurs to form a cyclic imidocarbonate which reacts with proteins in the immobilization process (Kohn and Wilchek, 1982). In the case of agaroses, where no vicinal hydroxyls are present, reaction with enzymes is via the cyanate ester. The method has been employed for the attachment of penicillin acylase (EC 3.5.1.11) to Sephadex G-200 for the commercial production of 6-aminopenicillanic acid from penicillin G (Lagerlöff *et al.*, 1976). A number of covalent attachment methods were tested and CNBr-activated Sephadex G-200 was chosen because it gave an enzyme preparation with good activity, coupling yield, stability, filterability and economical cost. The immobilized penicillin acylase has been used since 1973 for the production of 6-APA and has given over 100 reuses in a batch-stirred tank system, recovery of enzyme being effected by a filter press. It had also been used in a recirculation reactor in which substrate was circulated through a thin bed of immobilized enzyme and this system superseded the batch reactor because it overcame the problem of enzyme losses during enzyme recovery. It also avoided the microbial contamination of the penicillin acylase preparation which could result from handling the enzyme at the recovery stage and the possibility of enzyme inactivation by the proteolytic activity of the contaminants. The immobilized enzyme gave better product yield and purity with lower handling costs than the old process, which utilized *Escherichia coli* cell-bound enzyme (Lagerlöff *et al.*, 1976).

Kurkijarvi, Raunio and Korpela (1982) immobilized a commercially available bioluminescence reagent containing the luciferase and the NADH:FMN oxidoreductase from *Beneckea harveyi* on to CNBr-activated Sepharose 4B. This system was used in a continuous-flow column reactor for monitoring NADH at picomole levels by light-emittance measured in a luminometer. Some 400 measurements were made with a single column reactor without any decrease in sensitivity or accuracy and at least 40 times more analyses could be made with the immobilized system than was possible with the soluble reagent. The reusability of the system was limited by packing and disruption of the

support but more rigid supports (e.g. cross-linked agarose) were envisaged to overcome this problem. Agarose was found to be a particularly useful support as it has no light-absorbing characteristic at 490 nm.

Cellulose was one of the first materials to be used as an enzyme support and naturally occurring polysaccharides such as cellulose, starch, dextran, agarose have been widely used because they are readily available and readily derivatized. Carboxymethyl cellulose can be converted to the hydrazide (and in this form is commercially available as Enzite) which needs only to be converted to the azide derivative, by reaction with nitrous acid, prior to reaction with enzyme. This was one of the earliest methods developed (Mitz and Summaria, 1961). Aminoaryl derivatives of cellulose (e.g. *p*-aminobenzyl cellulose) can also be diazotized and reacted with enzymes. Polysaccharides can also be activated by cyanuric chloride or its dichloroderivatives such as 2-amino-4,6-dichloro-*S*-triazine or by triazinyl dyes such as Procion Brilliant Orange MGS (Kay and Crook, 1967; Sharp, Kay and Lilly, 1969). Cellulose derivatives are finding an application as paper strips to which enzymes can be bound for use in clinical assays. Peroxidase coupled to CM-cellulose has been used to measure hydrogen peroxide (Weetall and Weliky, 1966) and lactase bound to a triazinyl derivative of DEAE-cellulose has been used to measure lactose in the faeces of cancer patients (Stasiw, Patel and Brown, 1972). Despite the versatility of polysaccharide supports, emphasis has been placed on finding alternative enzyme supports because the polysaccharides are susceptible to microbial degradation, need extensive washing to remove physically adsorbed materials and show attrition and compression when used in stirred and packed-bed reactors.

An early development was poly-*p*-amino polystyrene (Grubhofer and Schleith, 1953) which was activated by diazotization. The polymer is available commercially and can be activated by phosgene and thiophosgene but it has not been widely used, possibly because of its hydrophobic nature. Ethylene–maleic anhydride and methyl vinyl ether–maleic anhydride copolymers, which are commercially available, have been used for enzyme immobilization. The physical form of these polymers is not well suited to large-scale use, although it can be improved by cross-linking with a diamine, e.g. diaminohexane. Smith (1981) attached a hydrophobic side chain to vinyl ether–maleic anhydride copolymers and used the modified support for the immobilization of penicillin acylase. Recovery of the immobilized enzyme was effected in a water-immiscible solvent (*n*-decanol) which was added to the reaction mixture. This technique allows the use of very small particles which would be difficult to recover by physical techniques.

Inman and Dintzis (1969) described the use of derivatized polyacrylamide, and aminoethyl and hydrazide derivatives are commercially available as Bio-Rad Aminoethyl and Hydrazide Bio-Gel P. Copolymers of acrylamide and derivatized acrylamides are also commercially available as Enzacryl AA, AH, Polythiol and Polyacetal. A further derivative of polyacrylamide is the bead polymer formed from methacrylamide with allyl glycidyl ether (oxirane) groups as the reactive components. This system has been reported for the immobilization of a number of enzymes including penicillin acylase (Kramer *et*

al., 1976, 1981) and β-galactosidase (EC 3.2.1.23) (Hannibal-Friedrich, Chun and Sernetz, 1980). It is commercially available as Eupergit.

A number of papers have described the use of nylon as a support. It is commercially available as powders, tubes, hollow fibres and spun-bonded films and is mechanically strong and not subject to microbial degradation. Because of its inertness, nylon requires to be activated and this can be achieved by mild acid hydrolysis to partially degrade the polymer and expose free amino and carboxyl groups which can then be activated by one of many techniques, e.g. conversion of carboxyl groups via hydrazine and subsequent diazotization or activation of amino groups by glutaraldehyde. Alternatively, reactive groups can be introduced by *O*-alkylation or *N*-alkylation of the peptide chains. *O*-Alkylation has been carried out using dimethyl sulphate and by reaction with triethyloxonium tetrafluoroborate (Campbell, Hornby and Morris, 1975; Morris, Campbell and Hornby, 1975). The resulting imidate salt can then be coupled directly with enzyme, or a spacer arm can be introduced such as diaminohexane to which enzyme can be coupled. Although this technique could have application for small-scale use, its complexity and the need to use a toxic chemical (dimethyl sulphate) might limit its use for large-scale immobilization of enzymes. *N*-alkylation can be achieved by a four-component condensation between the amino and carboxyl groups on the support, and an aldehyde and an isocyanide following mild acid hydrolysis, and in its most usual form results in a structure with isocyanide functional groups (Goldstein and Manecke, 1976). Recently Salleh (1982) has shown that nylon tubes can be activated by a high-temperature (90°C) treatment with glutaraldehyde. Studies with glucose oxidase showed a higher coupled activity and yield for enzyme bound by polyethylene imine and glutaraldehyde to the activated tubes than for material activated with triethyloxonium salts. These techniques have found application in the analytical field. Sundaram (1979) has developed a disposable nylon pipette tip to which enzymes can be bound (the 'Impette'). Substrate is drawn into the tip, allowed to react for a given time and then expelled and the amount of product determined. The results obtained have been promising and it is felt that this device will be of value where the expense of autoanalyser systems is not justified, e.g. for use in research laboratories, in small clinics, and by physicians in private practice. For autoanalyser systems, enzymes immobilized on the inside surface of nylon tubes have been used very successfully. This technique has been used for glucose determinations using glucose dehydrogenase (EC 1.1.1.47) (Hornby, Noy and Salleh, 1977) and an aliquot of enzyme could be used for 100 times as many determinations as the free enzyme. Mazid and Laidler (1979) co-immobilized NAD and yeast alcohol dehydrogenase to the inside of nylon tubes and used this system for measuring ethanol over a period of one month without detectable activity loss. The coenzyme was recycled using phenazine and thiosulphate and 2,6-dichlorophenol indophenol. As co-enzymes are often as expensive as enzymes, the value of this work can be appreciated.

A technique that seems to offer the possibility of a wide application is the covalent attachment of enzymes within polyurethane foams (Wood, Hartdegen and Hahn, 1982). The enzyme is covalently bound by reaction with isocyanate groups.

Although the majority of papers concerned with covalent attachment techniques refer to organic supports, there has been increasing interest in the use of inorganic supports such as carbon, metals, ceramics, silica and alumina. The advantage of such materials is their mechanical strength, resistance to microbial attack, good hydraulic property and ease of regeneration. Many can be made into rigid porous structures, and this is necessary with some inorganic powders which have poor physical and hydraulic properties. A comprehensive review of the use of inorganic supports has been provided by Kent, Rosevear and Thomson (1978) who concluded that these will increase in importance as their properties are improved and costs reduced. One of the most widely used materials has been controlled-pore glass which is produced by the leaching out of the boric-acid-rich phase which separates during the heat treatment of certain borosilicate glasses. The resulting silica-rich porous glass can be activated by treatment with γ-aminopropyl-triethoxy silane to which the enzyme can be immobilized by methods applicable to any amine-bearing support (Weetall and Filbert, 1974). The high cost of the support has precluded its large-scale use. In addition, gradual leaching of silica, particularly at alkaline pH, has been noted. This has been improved by impregnation of the glass by zirconium oxide, but this made the support even more expensive and has resulted in the development of cheaper and more stable porous ceramics made from silica, alumina and titania (Messing, 1974a,b). Macroporous supports (titania, calcium phosphate, alumina) have also been prepared by the Harwell group (Kent, Rosevear and Thomson, 1978). Talbot *et al.* (1982) compared two fluidized-bed reactors in which a parathion hydrolase was immobilized to porous glass and to activated alumina. Their interest was in developing an effective detoxification system for water containing pesticides. The denser alumina support was capable of use at high flow rates whereas the glass was lost from the column. It was also cheaper (US$0·50 per kg of activated alumina compared with $800 – $1600/kg of porous glass). The glass-supported enzyme was better at higher substrate concentrations, possibly because of reduced diffusional limitation of enzyme within the pores of the glass. However, as the level of pesticides in waste water is low, this was not an advantage for this application. In addition, the glass-supported enzyme showed less sensitivity to inhibition by salts and solvents than the alumina-bound enzyme, again probably by virtue of the greater diffusion limitation effects with the porous glass. Both reactors retained 80–85% of their initial activity after 160 days of intermittent use. These data indicate that the system of choice would undoubtedly be the cheaper alumina-bound enzyme.

A cheap porous silica-gel carrier to which enzyme was coupled after silanization was reported by Roy and Kundu (1979).

Kvesitadze and Dvali (1982) reported good coupling yields for fungal and bacterial α-amylases (EC 3.2.1.1) on porous silica carriers using titanium salt activation. Calcium chloride was added to the immobilization mixture to reduce the exchange of calcium with hydrogen groups on the silica which results in enzyme activity loss. These workers proposed the use of columns of immobilized α-amylase and glucoamylase to provide pure glucose from starch for medical application such as intravenous injection.

Several papers have reported the immobilization of enzymes to various

forms of carbon. Because of their electrical conductance property, these materials offer application in the analytical field for the production of enzyme sensors. They are relatively inexpensive and are available in a number of forms, including electrodes. Cho and Bailey (1979) used activated carbon for immobilizing glucose oxidase and found that by providing a catalytic surface for decomposition of hydrogen peroxide the support reduced the inactivation of the enzyme by the peroxide produced during the oxidation of glucose. Bourdillon, Bourgeois and Thomas (1980) attached glucose oxidase to carbon electrodes using carbodimide activation (*see also* Aston and Turner (1984) and Neujahr (1984), in Volume 1 of this Series).

Carbon has to be oxidatively pretreated to provide surface-functional oxygen groups. Osborn *et al.* (1982) used radio-frequency oxygen plasma and electrochemical oxidation in order to obviate contamination of the surface with metal ions, which is found with many traditional methods such as dichromate or permanganate treatment. Cyanuric chloride and carbodi-imide were used for coupling α-chymotrypsin (EC 3.4.21.1), α-chymotrypsinogen and glucose oxidase (EC 1.1.3.4) to granular activated carbon, the former being found to be the better method.

The amount of enzyme attached to a solid is obviously related to the surface area available. The problem with using very small particles which offer a large surface area is their recovery from the reaction medium. One way of overcoming this has been to increase the porosity of larger particles, but this has led to diffusional limitation effects which limit the effectiveness of enzyme located deep inside pores. This in turn has led to the development of methods to retain enzyme at the outer edge of a porous particle and to the use of pellicular supports in which an enzyme-coupling phase is attached to an inert core. Magnetic supports offer an attractive alternative in that very small particles can be used because of the high efficiency of magnetic separation (Halling and Dunnill, 1979, 1980). Magnetic supports may be used where particulate fouling is a problem, because of the ease of separation and recovery.

Brief mention has been made of titanium-salt activation for enzyme immobilization. Barker, Emery and Novais (1971) reported on the development of this versatile technique which is applicable to organic and inorganic supports. It consists of the steeping of the support in a transition metal salt solution (e.g. $TiCl_4$) followed by recovery of the activated support which is washed well and then reacted with enzyme. In a modification of this technique (Cabral *et al.*, 1983), α-amylases and invertase were immobilized using glutaraldehyde on titanium-activated porous glass beads which had been aminated by reaction with diaminohexane. Very good operational stability was reported for these preparations.

ENTRAPMENT TECHNIQUES

Matrix entrapment

Like adsorption procedures, entrapment processes are relatively simple to carry out. The basic technique involves the occlusion of enzyme within the

lattice structure of a polymer matrix and this is usually effected by mixing enzyme with appropriate monomers and initiating a polymerization reaction. A number of materials have been used including polyacrylamide, polyvinyl pyrrolidone, starch, silicon resin, κ-carrageenin, collagen, alginates and epoxy resins. One of the most widely used procedures has been immobilization in polyacrylamide gels, first reported by Bernfeld and Wan (1963). In this technique enzyme is mixed with acrylamide and *N,N'*-methylene *bis* acrylamide (the cross-linking agent) and polymerization is initiated by the addition of potassium persulphate or riboflavin. Because the reaction is exothermic, cooling is required to minimize thermal denaturation of the enzyme. If polymerization is initiated by X- or γ-irradiation, the reaction can be carried out at low temperatures (Maeda, Yamauchi and Suzuki, 1973) but the cost is high and the reaction entails the use of specialized equipment. This has reduced the usefulness of this technique for large-scale application. A disadvantage of forming a gel is the need to granulate it to provide a useful physical form of the enzyme. An alternative method, in which enzyme and monomer are dispersed in a hydrophobic phase (toluene and chloroform) and the polymerization is carried out under nitrogen, results in spherical gel beads (Dahlquist, Mattiasson and Mosbach, 1973). One application of gel-entrapped enzymes has been to prepare enzyme electrodes in which a thin layer of immobilized enzyme is associated with either a potentiometric (e.g. ion-selective electrode) or an amperometric sensor (e.g. oxygen electrode). One of the earliest enzyme-sensors was a glucose probe consisting of glucose oxidase entrapped in a polyacrylamide gel associated with an oxygen electrode (Updike and Hicks, 1967). Several glucose-measuring devices have been described utilizing glucose oxidase and glucose dehydrogenase. More recently Liu, Weaver and Chen (1981) described a probe consisting of glucose dehydrogenase immobilized in a polyacrylamide gel cast over the surface of a platinum screen; however, poor reusability and storage life limited its effectiveness. Devices based on conventional electrodes and entrapped enzymes are not easy to construct. Trevan (1980) commented on the difficulty of securing a gel-entrapped enzyme to a round-bottomed electrode. The presence of the gel imposes a diffusional barrier, both to the penetration of substrate to the enzyme and to the passage of product to the sensor. A direct attachment of enzyme to the electrode would appear to be preferable because diffusional limitations are reduced. A major disadvantage of entrapment procedures is the physical loss of enzyme from the matrix. In order to obviate this, a tight gel is needed and this results in severe diffusional limitation, particularly with substrates of high molecular weight.

Matrix entrapment techniques have found their major application for cell immobilization and several reviews of methodology and immobilized-cell systems exist (Abbott, 1978; Cheetham, 1980; Kolot, 1980, 1981a,b; Mosbach, 1983). Large-scale processes utilizing immobilized cells for the production of L-aspartic acid and L-malic acid have been described by Chibata (1978). Interest in immobilized-cell systems has grown rapidly, particularly as they would appear to provide the means for carrying out multi-enzyme processes, particularly biosynthetic reactions, without the need to isolate and immobilize individual enzymes. In addition, they provide cofactor regeneration systems.

A more specialized form of enzyme entrapment is the use of microencapsulation whereby enzyme is retained within a thin-walled droplet.

Microencapsulation processes

The immobilization of enzymes within semi-permeable microcapsules is a technique that has been pioneered by Chang (1976a,b). Two basic processes can be used. In the first process a polymer (e.g. cellulose nitrate) is allowed to separate out around enzyme microdroplets produced by agitating well an aqueous dispersion of enzyme in a water-immiscible solvent containing the polymer in solution. This physical-separation technique is termed coacervation. This technique has been used with ethyl cellulose, nitrocellulose, polystyrene, polyethylene, polyvinyl acetate, polymethylmethacrylate and polyisobutylene (Chibata, 1978). In the second technique, termed interfacial polymerization, an aqueous solution of enzyme and monomer is dispersed in a water-immiscible solvent. The second (hydrophobic) monomer is then added, dissolved in the solvent, and polymer is formed by chemical reaction at the interface of the microdroplets. Although this system has been used mainly for nylon, other polymers such as polyurethane, polyester and polyurea have been described (Chibata, 1978). Haemoglobin is usually co-encapsulated with the enzyme to provide a buffered and stabilizing environment (Chang, 1976a). Further stabilization of the enzymes can be effected by cross-linking with a bifunctional reagent.

These procedures are relatively sophisticated but a large-scale microencapsulation process has been described by Dinelli, Marconi and Morisi (1976). In this technique an aqueous enzyme solution is emulsified in a water-immiscible solvent containing a fibre-forming polymer such as cellulose triacetate. The mixture is then extruded through a spinneret into a coagulant which precipitates the polymer as fibres in which enzyme is entrapped in microdroplets. The fibres can be cut up and used in stirred reactors, but more generally they are used in column reactors which can be operated continuously or as recycle reactors. A plant utilizing fibre-entrapped β-galactosidase for hydrolysing lactose in milk has been reported to be in operation (Pastore and Morisi, 1976). The method has been applied to a range of enzymes including penicillin acylase, glucoamylase (EC 3.2.1.3), glucose isomerase (EC 5.3.1.5), invertase (EC 3.2.1.26), aminoacylase (EC 3.5.1.14) and tryptophan synthase (EC 4.2.1.20). A possible limitation to its general use is the need for specialized equipment and know-how to prepare the fibres.

DISCUSSION

The foregoing has illustrated the diversity of methods for enzyme immobilization. Composite systems, utilizing two or more of the basic methods, have also been used. For example, covalently immobilized or cross-linked enzyme preparations have been microencapsulated. One system that is difficult to classify within the proposed system is the use of ultrafiltration devices in which enzyme reacts in solution within the reactor but is retained by an appropriate

filter. Such a system is best suited to stable enzymes but has the advantage that high-molecular-weight substrates can be utilized. However, problems attributable to enzyme inactivation and leakage through the ultrafiltration membrane have limited the usefulness of such devices. All of the immobilization procedures have advantages and disadvantages relative to one another and the choice for a particular application will depend upon the constraints imposed. For example, large-scale applications will require a readily available support and a relatively simple and inexpensive coupling procedure which can be used with an impure enzyme to give an active stable immobilized enzyme. Activity may be traded on for improved stability. Physical stability in stirred reactors is important, although for column systems poor hydraulic characteristics can be overcome by the use of thin-bed reactors. Stability is particularly important for analytical devices, especially where in-vessel monitoring systems are being devised. One of the most heavily constrained areas is that of enzyme therapy, particularly where the enzyme is to be injected into the bloodstream.

In the next section the effects of immobilization, both on the usefulness of enzymes and on their properties, are discussed.

The effects of enzyme immobilization

INTRODUCTION

The effects of immobilization processes can be discussed under two major headings. In the first case the development of procedures for immobilizing enzymes has increased the usefulness of enzymes for a number of applications. Enzymes have been used for many years in industrial processing, food processing and for analytical and therapeutic purposes. However, the use of soluble enzymes is limited. They are not easily recovered for reuse, they can be used only in stirred batch-operated reactors and, if their removal from the process stream is required, specialized and often expensive treatment is required which results in yield losses. For therapeutic purposes, enzymes often need to be administered in large amounts to combat their removal and inactivation, and this in turn can provoke unwanted immune responses in the patient. These disadvantages can be obviated by the use of immobilized enzymes. In addition, the potential exists for the fabrication of specialized enzyme reactor systems such as enzyme probes and extracorporeal devices.

However, by the process of immobilization an enzyme is converted from a homogeneous catalyst to a heterogeneous catalyst and this can have profound effects on the properties of the enzyme. The activity, stability, pH and temperature optimum, kinetic properties and substrate specificity can be altered by the association with a support.

These two effects are discussed in the following section.

APPLICATIONS FOR IMMOBILIZED ENZYMES

Industrial applications

For an immobilized enzyme to be established for carrying out an industrial process it must successfully compete with chemical procedures and with soluble

enzyme processes in terms of economy. This may accrue from improved product yield and purity, lower capital and labour costs, compactness of plant, flexibility, better process control and lower running costs. The cost of the immobilized enzyme will be related to the cost of the product but, in general, the lower the cost, the fewer reuses will be needed to make the process economical in enzyme-cost terms; hence, the simpler the coupling procedure and the cheaper the support, the better. Where food processing is concerned, restrictions will be imposed limiting the chemicals that can be used on toxicological grounds.

At least three large-scale processes using immobilized enzymes are in operation at present. These are the production of amino acids by enzymic resolution using an aminoacylase, the production of 6-aminopenicillanic acid by the dephenylacetylation of benzyl penicillin G by penicillin acylase, and the production of fructose syrups from glucose using glucose isomerase. Of these, the fructose syrup production is the largest single application of an immobilized enzyme in the world with an estimated potential market for 1985 of $6{\cdot}8 \times 10^9$ lb ($\approx 3{\cdot}1 \times 10^9$ kg) (Chen, 1980a,b). This would appear to be disappointing progress in view of the earlier hopes for immobilized enzymes but Poulsen (1981, 1984) has commented on the factors apart from technical success which have to be satisfied to ensure the successful development of an industrial process based on immobilized enzymes, and he considered that success was doubtful if a cheap soluble enzyme process was available or if an established chemical process had operated for some time. He felt that more complex processes, particularly employing immobilized cells, offered greater potential for the future. Van Beynum (1980) had also expressed this view and felt that the potential for immobilized enzyme systems lay in novel applications and the development of new products.

The use of lactase to remove lactose from milk and to produce a sweet syrup from whey has attracted a great deal of research and has been reviewed by Greenberg and Mahoney (1981); *see also* Moulin and Galzy (1984) in Volume 1 of this Series. The cost of the enzyme has made an immobilized enzyme process attractive. However, despite small plant operations in Italy, France, Finland and in Britain, a number of economic and practical constraints have yet to be overcome before the process can be scaled up (Poulsen, 1981). Enzyme longevity needs to be improved and problems due to fouling of the support need to be overcome. It has been suggested that fouling of the supported enzyme by the milk and whey solids could be overcome by the use of magnetic support-bound enzyme (Halling and Dunnill, 1980). Friend and Shahani (1982) described a regenerable system based on an *Aspergillus oryzae* enzyme immobilized on a derivative of cross-linked Sepharose but the high cost appeared to preclude the use of this system and the authors considered that the better system would be a physically adsorbed enzyme capable of regeneration by physical methods; this would have the advantage, not only of cheapness but also of complying with the regulations governing the supports and chemicals which can be used where the product is used for food processing. The problem of microbial contamination of lactase reactors was discussed by Harju (1977) who commented on the potential use of co-immobilized glucose oxidase

to produce H_2O_2. Greenberg and Mahoney (1981) noted that although immobilized-lactase technology was well advanced, its application was as much dependent upon economic, environmental and political considerations as on technical expertise.

Papain (EC 3.4.22.2) is used in beer production to prevent the formation of 'chill-hazes' which result from the precipitation of protein–polyphenol or protein–tannin complexes. The enzyme is expensive and it is possible that future legislation could restrict its use; hence the current interest in developing an immobilized enzyme process. However, the enzymic reaction is slow and alternative procedures could well replace this process eventually (Brocklehurst, Baines and Kierstan, 1981). The need for an immobilized enzyme process for the depectinization and clarification of apple juice was identified by Kilara (1982) in a review of the role of enzymes in the processed apple industry.

Chibata (1978) has reviewed the investigations carried out by Tanabe Seiyaku Co. Ltd. into the use of immobilized aspartase (EC 4.3.1.1) for the production of L-aspartic acid from fumaric acid. The cheapness of aspartic acid precluded the use of an immobilized enzyme system and an immobilized *Escherichia coli* cell system was found to be more cost effective. Immobilized-cell systems were also developed for L-malic acid production and for urocanic acid manufacture using *Brevibacterium ammoniagenes* and *Achromobacter liquidum* respectively. In both cases unwanted side-reactions had to be suppressed to prevent yield losses (Chibata, 1978).

Schmid (1979) reviewed the potential importance of oxidoreductases, a group of enzymes that outnumber most other classes of enzymes. He noted that while several are commercially available for analytical use only two, glucose oxidase and catalase, have been used for large-scale processes. This has been attributed to the cost of isolating the enzymes, their relatively low stability and the need in many cases to provide expensive cofactors. However, they are valuable for carrying out a number of chemical modifications such as the hydroxylation of steroids, antibiotics and terpenes and the oxidation of alkanes. Extensive development of cofactor regeneration systems is in progress. Over one-third of the *c.* 2000 listed enzymes requires one of the adenine nucleotides, NAD, NADP, ATP, FAD and coenzyme A (Mosbach, 1982). Their high cost requires that a system for recovering and recycling them is developed. In order to immobilize cofactors they need first to be derivatized to provide functional groups for attachment to a support. An alternative system for retaining unmodified cofactors in lipid–polyamide microcapsules has been described (Yu and Chang, 1982). Enzymatic regeneration procedures appear to be favoured. One problem associated with cofactor regeneration is that the substrate and product of the regeneration system may be difficult to separate from the product of the cofactor-requiring enzyme. A system for regenerating NADH by the oxidation of ammonium formate to CO_2 using formate dehydrogenase (EC 1.2.1.43) does not suffer this disdavantage as the product is easily removed from the reaction (Wichmann *et al.*, 1981; Wandrey, Wichmann and Jandel, 1982). Klibanov and Puglisi (1980) have described a system applicable to several cofactors in which reduction is effected by hydrogen catalysed by the hydrogenase (EC 1.18.3.1) activity of immobilized

Alcaligenes eutrophus cells. No undesirable products are formed as in the previous method. The use of an immobilized hydrogen dehydrogenase (EC 1.12.1.2) has also been described by Danielsson *et al.* (1982). A limitation of such systems for large-scale use would seem to be the need to carry out the reaction under hydrogen. The field of cofactor regeneration has been reviewed by Lowe (1981). At present, commercially feasible systems have not been developed but the state of present research would indicate that this will be accomplished in the near future.

Immobilized enzymes may find application in the production of fragrances, the world market for which is estimated at US $\$2 \times 10^9$ annually (Schindler and Schmid, 1982). Enzymic processes for terpene ester racemization are being developed at present.

Cellulose is a plentiful energy source and the feasibility of producing glucose via the enzymatic hydrolysis of cellulose by cellulase is being actively pursued in many countries (Eveleigh, 1983). The enzyme reaction is slow and this may preclude the use of an immobilized enzyme although the use of soluble or colloidal cellulose may improve the process (Chibata, 1978). A process for ethanol production, which has a saccharification process linked to an immobilized-yeast reactor, has been developed to pilot-plant scale (Eveleigh, 1983).

The production of invert sugar from sucrose using immobilized invertase appears technically feasible but economically it is not able to compete with the glucose-isomerization process (Chibata, 1978). Fructose production from inulin, a carbohydrate reserve present in some plants, is being investigated because of the potential for producing syrups with a higher fructose content (>75%) compared with the isomerase process (40–55%) (Guiraud *et al.* 1983). The development of immobilized pesticide-detoxification enzymes for the treatment of waste water is at the research stage, but the potential for a cheap reactor system exists (Talbot *et al.* 1982). These workers described the use of immobilized parathion hydrolase to degrade the pesticide parathion to *p*-nitrophenol and diethylthiophosphoric acid.

One of the major constraints on the commercial application of enzymes is the limited number available in commercial quantities. Klibanov and co-workers at the Massachusetts Institute of Technology are trying to extend the range of applications for those enzymes currently produced in commercial amounts. Alberti and Klibanov (1982) used glucose oxidase from *Aspergillus niger*, a fairly stable, inexpensive and readily available enzyme, to oxidize glucose in the presence of benzoquinone (replacing oxygen as electron accepter). The product, hydroquinone, is commercially important as a photographic developer and as an inhibitor of auto-oxidation and polymerization and was produced in nearly 100% yield. Enzyme covalently attached to alumina and packed in a column was used for two weeks with no measurable reduction in its efficiency. Glucose oxidase is extremely specific for glucose (hence its analytical use for glucose measurement) but is less specific for the second substrate and this fact has been used to advantage by Alberti and Klibanov. An acetone–water mixture was needed to obtain a reasonable concentration of benzoquinone (0·1 M). It was speculated that the gluconolactone produced could be hydrolysed to gluconic acid or could be enzymically

converted to 2-keto-D-gluconic acid which could be converted to the industrially useful chemical furfural. The economics of the process compared with alternative methods for hydroquinone were not discussed. The intention of the work was to demonstrate a novel application for a readily available enzyme that might have some commercial value.

Klibanov and co-workers have also demonstrated steric selectivity effects. Pelsey and Klibanov (1983) showed that a sulphatase from *Helix pomatia*, an inexpensive and commercially-available enzyme, could hydrolyse β-naphthyl sulphate to produce the commercially important β-naphthol while α-naphthyl sulphate was not affected by the enzyme due to steric hindrance by the benzene ring adjacent to the one bearing the sulphate group. Klibanov and Siegel (1982) reported the hydrolysis of *trans* isomers of methyl-3-arylacrylates by porcine liver carboxylesterase (EC 3.1.1.1) to be an order faster than hydrolysis of the *cis* isomers. Klibanov and Giannousis (1982) found that xanthine oxidase (EC 1.2.3.2) oxidized the *trans* isomer of β-arylacroleins, cinnamaldehyde and β-(2-furyl) acrolein two orders of magnitude faster than the *cis* isomers.

Klibanov *et al.* (1977) described the synthesis of *N*-acetyl-L-tryptophan ethyl ester from acetyl-L-tryptophan and ethanol in a biphasic system by chymotrypsin coupled to porous glass. In a totally aqueous system the reaction will not proceed and ester hydrolysis is favoured, but by using a water-immiscible solvent system the equilibrium is switched to favour the synthesis. This technique represents a new approach to preparative organic synthesis in that it permits the reversal of the equilibrium of reactions in which water is one of the products.

Klibanov, Berman and Alberti (1981) reported the directed hydroxylation by oxygen of L-tyrosine, D-(−)*p*-hydroxyphenylglycine and L-(−)phenylephrine in the presence of dihydroxyfumaric acid as a hydrogen donor using horseradish peroxidase (EC 1.11.1.7) at 0°C to produce L-dopa, D-(−)-3,4-dihydroxyphenylglycine and L-epinephrine respectively.

It seems likely that the use of enzymes for industrial processes will increase as more become available and as their versatility is elucidated. Immobilization procedures will help to establish enzymic processes because they can provide a reusable catalyst, separable from product and capable of being incorporated into appropriate reactor systems. Their future would seem to lie in the area of relatively small specialized processes where chemical techniques may be costly or difficult to carry out because of the need to use toxic reagents.

Analytical applications

Enzymes have been extensively used for carrying out analyses because of their specificity and because of the range of reactions that they catalyse (Bergmeyer, 1974). One of the advantages of using immobilized enzymes is the potential to reduce costs by reusing enzyme. This has become increasingly important in clinical chemistry where the number and range of analyses required, and hence cost, has increased. To obviate this problem immobilized enzyme systems based on the flow-through autoanalyser units commonly used in clinical laboratories are being developed. Enzyme is immobilized to the inner wall of

tubing (nylon is preferred) and the enzyme reactor is placed in series with an appropriate detector. This type of system appears well suited to the demands of a clinical laboratory (50–60 assays per hour). Sundaram, Blumenberg and Hinsch (1979) reported a 50-fold reduction in assay cost using an immobilized glucose dehydrogenase nylon tube reactor for glucose measurement. Based on the same principle, assays have been developed for urea, glucose, uric acid, pyruvate, lactate, citrulline, creatine, creatinine, glycerol and cholesterol (Sundaram, 1982). Three commercial devices for glucose estimation described by Keyes, Semersky and Gray (1979) use the flow-through principle. A nylon coil to which hexokinase (EC 2.7.1.1) and glucose-6-phosphate dehydrogenase (EC 1.1.1.49) are immobilized is marketed by the Technicon Corporation. It is used in an autoanalyser set-up and glucose is estimated by the rate of formation of NADH which is determined using an in-line spectrophotometer. Because the storage stability of the reactor is good and activity losses occur mainly during its operation it may be used effectively in small laboratories with a few samples to analyse daily (Wease, Anderson and Ducharme, 1979). These workers used one enzyme coil for carrying out over 2000 assays over a period of 91 days, the reactor being stored at 4°C for half of this time. In the Kimble analyser, glucose oxidase immobilized to a porous inorganic support and held in a column is used to measure glucose. The detector is a tri-electrode system, with a membrane to exclude interfering substances, which measures the H_2O_2 produced by the enzyme reactions. A similar device is used in the Leeds and Northrop instrument.

A simple device based on enzyme immobilized to the inner surface of a nylon tube linked to an automatic pipette ('Impette') has been described by Sundaram (1979). The sample is sucked into the enzyme tube, held for a time and then expelled. The level of product in the sample is then determined. The system has been used for urea, glucose, uric acid, amino acids and pesticides (Sundaram, 1979) and could have an application where automated systems are not justified.

In the systems described previously the enzyme reactor and the detector have been physically separate entities. However, one advantage of the development of immobilization techniques has been the potential for directly linking the enzyme to the sensor to produce an enzyme probe or enzyme sensor. The development and application of such devices has been reviewed by Barker and Somers (1976), Carr and Bowers (1980) and Pinkerton and Lawson (1982) and it is a rapidly expanding field. Sensors incorporating not only immobilized enzymes but also immobilized microbial cells have been developed. Karube and Suzuki (1983) describe microbial sensors for measuring BOD, ammonia and glutamic acid; *see also* Chapter 11 of this volume. A variety of sensors has been used including pH electrodes, ion-selective electrodes, oxygen electrodes, gas-sensing electrodes, thermistors and light-sensing devices. An example of the last is the flow-through column containing luciferase (EC 1.14.99.21) and NADH:FM oxidoreductase immobilized to cyanogen bromide-activated Sepharose 4B installed within a luminometer used to measure picomole levels of NADH (Kurkijarvi, Raunio and Korpela, 1982). Despite some physical disruption of the support the system was used for 400

consecutive determinations. The potential exists for linking this system to other enzyme reactions generating NADH. Enfors and Nilsson (1979) described a penicillin electrode based on β-lactamase (EC 3.5.2.6) entrapped within a semi-permeable membrane at the base of a pH electrode. Unfortunately conditions necessary to give a short response time, i.e. a thin enzyme layer, resulted in a short life-time for the probe of 1–2 days which would be unsatisfactory for in-vessel monitoring of fermentations. In addition, because buffer-capacity changes in the medium affect sensitivity and preclude pre-calibration of the probe, it is not yet suitable for direct fermentation monitoring. Lowe, Goldfinch and Lias (1983) immobilized β-lactamase and bromocresol green to a transparent cellophane membrane and placed the enzyme membrane between a light source and a detector. Protons produced by the enzyme reacting on penicillin G reacted with the dye and this was detected by an increased voltage from the photodiode detector. The system showed good stability over a year and could be constructed either as a flow cell or as a probe. Similar devices were prepared to measure glucose and urea using glucose oxidase/bromocresol green and urease/bromothymol blue respectively, although stability was not as good.

Many enzyme reactions are exothermic and the heat evolved can be used to measure the rate of reaction. The system most commonly used is an immobilized-enzyme column reactor containing a thermistor. Enzyme thermistors have been reviewed by Mosbach, Mandenius and Danielsson (1983) who comment on their potential for process control and fermentation analysis.

Several assay systems have been devised using ion-selective electrodes but a problem with such systems is the specificity of the electrodes. The use of gas-sensing membranes and air-gap electrodes has been preferred because of their freedom from interference.

An enzyme sensor with lysine decarboxylase (EC 4.1.1.18) immobilized on a carbon dioxide gas-sensing electrode has been described for assaying L-lysine in an amino-acid mixture (Tran, Romette and Thomas, 1983). Recent interest has now turned to the development of enzyme sensors using ion-selective field-effect transistors which offer the potential for miniaturization. Enzymes can be coupled to pH- or gas-sensing field-effect transistors (FEDs). β-Lactamase, immobilized by cross-linking with albumin and layered over a silicon nitride pH-sensitive FED, has been used for penicillin determinations (Caras and Janata, 1980). These devices are still in their early stages of development, but they offer exciting possibilities, particularly for *in vivo* monitoring devices (Pinkerton and Lawson, 1982; Danielsson *et al.*, 1983). One of the most desirable enzyme sensors is the glucose probe. This is important because of its potential application in fermentation monitoring where glucose is often used as a carbohydrate source. In addition, devices for monitoring blood glucose are being developed with one ultimate objective: of producing an implantable electrode for *in vivo* monitoring; such a system could be useful in warning diabetic patients of impending hypoglycaemia. Many devices have used glucose oxidase, which catalyses the oxidation of glucose using oxygen as an electron acceptor; the dependence upon oxygen can be a problem. Enfors (1981) comments on the low oxygen levels common in

fermentation broths. He describes an enzyme probe where, as the oxidation by glucose oxidase proceeds, oxygen is generated electrolytically. An elegant solution recently described is the use of glucose oxidase covalently linked to a carbon electrode on to which dimethylferrocene has been deposited (Higgins, Hill and Plotkin, 1983). The dimethylferrocene acts as an electron acceptor and renders the probe independent of oxygen.

Although a great deal of development work is being undertaken in the biosensor field, not many commercially viable systems have as yet been produced. Nevertheless, it is anticipated that more systems will emerge as problems of stability, cost, reproducibility, ease of manufacture and use are overcome.

Medical applications

Up to now the therapeutic application of enzymes has been limited because of the ease with which they are inactivated and removed from the body and because of their ability as foreign proteins to elicit immune responses from the host. These problems are now being overcome by the use of immobilized enzymes. These can either be administered *in vivo* or can be incorporated into an extracorporeal device similar to an artificial kidney machine. Thus far experimentation has been confined mainly to experimental animals and systems have been investigated for the treatment of enzyme-deficiency diseases (e.g. acatalasaemia using immobilized catalase, EC 1.11.1.6) and for the removal of growth substrates for some tumours (e.g. asparagine using immobilized asparaginase, EC 3.5.1.1). Adsorbants can also be immobilized with the enzyme to remove products of the enzyme reaction such as ammonia. For *in vivo* application much research has concentrated on the use of microcapsulated and liposome-entrapped enzymes. The preparation and application of immobilized enzymes for therapeutic purposes has been reviewed by several workers including Chang (1976a,b), Gregoriadis (1976a,b), Broun *et al.* (1977) and Chang (1977). Many problems remain to be overcome but the potential value of immobilized enzymes has been demonstrated.

EFFECTS ON ENZYME STABILITY, ACTIVITY AND KINETICS

The immobilization of an enzyme can have profound effects upon its activity, stability, substrate specificity and general kinetic properties. This has been discussed in many of the papers and reviews dealing with immobilized enzymes. A particularly readable overview has been provided by Trevan (1980). The immobilization process converts a homogeneous catalyst (soluble enzyme) into a heterogeneous catalyst in which enzyme is associated with a supporting or enveloping matrix. Enzyme activity is usually lost during the immobilization procedure, either because of the reactions involved in the process, which may denature the enzyme, or by occlusion of active sites in the enzyme–support complex. Minimal denaturation may be obtained by using an adsorption process for immobilization. However, the absence of strong binding links may be disadvantageous. The optimum chemical binding techniques are

those requiring mild conditions for the coupling reactions and effecting linkage by chemical groupings not associated with the active site of the enzyme. However, the support also can affect the enzyme by virtue of its hydrophobicity, porosity and charge. For enzyme in solution there is no diffusional limitation on substrate reaching the active site of the enzyme or on product leaving the active site. Michaelis–Menten kinetics are used to describe the relationship between substrate concentration and enzyme reaction rate. With immobilized enzymes, care has to be taken when applying Michaelis–Menten kinetics because of the effects of the support. For example, a raised K_m may result from substrate-diffusion limitation and may not result from a direct modification of the enzyme's active site. A similar argument may apply to the reduced effect of inhibitors on immobilized enzymes. It is not unusual to find that high-molecular-weight substrates are less easily converted than low-molecular-weight materials by immobilized enzymes. This has been noted with proteases and has been ascribed to the greater diffusion limitation of the larger molecules.

Diffusional effects are of two types — external diffusion limitation due to an unstirred layer (the Nernst layer) around the particles of enzyme, and internal or pore diffusion caused by substrate having to diffuse through the support matrix. External diffusion can be reduced by improving the movement of fluid around the particles, thus minimizing the thickness of the Nernst layer; thus, increasing stirring speed or substrate flow can be effective. In contrast, internal diffusion is unaffected by fluid flow around the particle but is related to the distance substrate has to diffuse to the enzyme's active site. For particulate supports this can be reduced by using small particles or by using a non-porous support (Halwachs, 1979). However, reduction in particle size can result in poor flow-through properties and can pose a problem, particularly for large-scale reactor systems. These are not insurmountable, as the use of thin-bed reactors has shown, but they increase the sophistication of the reactor. Non-porous supports, while advantageous, often have a lower enzyme-loading capacity. An alternative method is to effect immobilization in the outer regions of a porous particle. Enzyme thus immobilized will be more active than if it is evenly distributed throughout the particle (Do, Clark and Bailey, 1982). This can be achieved by stopping the adsorption process before enzyme has percolated too far within the particle. Alternatively, a pellicular support having a porous outer layer overlaying a non-porous core may be used. The advantage of increased activity resulting from restricting enzyme to the outer region of particles would be expected to be balanced by a greater rate of deactivation compared with systems with uniformly distributed enzyme (Do, Clark and Bailey, 1982). The best system will ultimately be a compromise between good activity and good stability.

In addition to diffusional limitation effects, the support can also exert partitioning effects due to its charge. In effect, an enzyme may be acting in a microenvironment very different from that pertaining in the bulk solution. For example, a negatively charged support will attract protons and this will result in a lower pH locally around the enzyme. The converse will apply for positively charged supports. The effects of these will be apparently to modify the

optimum pH of the enzyme. Similar attraction–repulsion effects will occur with charged substrates and products. Charge effects can be minimized by increasing the ionic strength of the reaction medium and it is important when characterizing immobilized enzymes to study their activity under a variety of ionic conditions (Dale and White, 1983).

Taken together, diffusional limitation and electrostatic effects can alter profoundly the properties of an enzyme. There can be an extension of the substrate range over which a linear response is obtained and this can be advantageous for analyses because dilution of sample may not be required (Carr and Bowers, 1980). Webb (1983) has noted that the matrix not only can restrict the diffusion of substrate to the enzyme, it may also (depending upon pore size) restrict the spatial rotation of the substrate to achieve optimal binding at the active site.

For simplicity, mathematical analyses of the effects of enzyme immobilization on reaction kinetics have not been included in this review. Detailed discussions of these by other more competent authorities can be found, such as those of Engasser and Horvath (1976) and Goldstein (1976).

One of the more controversial aspects of enzyme immobilization relates to its effect on enzyme stability. Although this desirable feature has been reported, it is not a general result of immobilization. Melrose (1971) noted that in only 30 of 50 reported immobilized enzymes had increased stability been reported. In addition, it is difficult to know when a true stabilizing effect has been achieved, because diffusional limitation effects can cause misleading impressions of increased stability. For example, diffusional limitation may result in only a proportion of enzyme within a bead being assayed. As activity decays, substrate can diffuse further into the bead and react with the enzyme present. The overall effect is to indicate no activity decay, which is not, in fact, the case. The effect of diffusional limitation on the apparent stability of glucose isomerase on immobilization has been analysed by Verhoff (1982). A mechanism for enzyme inactivation has been proposed by Mozhaev and Martinek (1982) which involves unfolding of the protein molecule and reaggregation, both intra- and intermolecular, to form inactive aggregates. It follows from this that enzyme immobilization by restricting conformational changes should improve enzyme stability. The importance of multipoint attachment (covalent or non-covalent linkages) to a conformationally suitable polymer (one with a surface geometry similar to the enzyme) for good thermostability was stressed by Martinek *et al.* (1977a,b). Immobilization of rat liver arginase to cyanogen-bromide-activated Sepharose 4B resulted in an immobilized enzyme that was markedly less affected by sodium dodecyl sulphate than the free enzyme (Muszynska and Wojtczak, 1979). This result was ascribed to the stabilization of the tetrameric structure of the enzyme. Gabel (1973) has shown that, as the number of attachment points of trypsin to cyanogen-bromide-activated Sephadex is increased, its stability to 8 M urea increases. The stabilization of proteases by immobilization, which prevents autolysis, does appear to be generally acknowledged. Some stabilizing effect may be found with charged supports. Barker (1982) has reviewed the stabilizing effect of polyelectrolytes.

That increased stability need not apply to all of the denaturing influences acting on immobilized enzymes is shown by the results of Yeung, Owen and Dain (1979). These workers immobilized a mixture of glycosidases with serum albumin using glutaraldehyde and cast the immobilized preparation in membrane form. Although resistance to thermal inactivation had increased, resistance to 8 M urea or low pH were unchanged.

Discussion

The development of enzyme-immobilization procedures has undoubtedly increased the practicability of using enzymes in a number of fields. In addition, enzyme-purification techniques are being improved and developed which will allow a wider range of enzymes to be made available. The range of microbial enzymes is being extended and work is in progress to widen our knowledge of the many biochemical transformations that are possible with micro-organisms (Rozazza, 1982). The potential uses for enzymes in organic chemistry are being actively investigated. Jones (1976) has predicted a steady increase in their application in the future. The development of commercially feasible cofactor-recycling systems will enhance progress in this area.

The immobilization procedures developed for enzymes have been applied to microbial cells. Although immobilized-cell systems offer some advantages, particularly for complex multi-step reactions and cofactor-requiring enzymes, they do have disadvantages. If live-cell systems are used, cell multiplication needs to be controlled to prevent break-up of the immobilized cell matrix. Side-reactions may need to be suppressed and their activity is low compared with that of immobilized enzymes, which results in a need for larger reactors. In addition, some leaching out of cells and cell contents may occur. Nevertheless, there is a great deal of research in progress to evaluate their potential applications: for example, their use for antibiotic production is being considered (Morikawa, Karube and Suzuki, 1979).

In the analytical field, enzymes have long been valuable, but the longer-term potential lies in the enzyme sensor, particularly for monitoring fermentation processes and for *in vivo* monitoring devices. For fermentation monitoring and control, a sensor is needed which is sterilizable, sensitive over the range needed, capable of easy calibration, reliable over long periods (several days) and robust. The device has to function in a complex medium which is constantly changing in composition, ionic strength, pH and oxygen level. With these constraints it is not surprising that such devices are not yet widely available: for a review of research in this field, particularly in Japan, *see* Chapter 11 of this volume.

Progress in the medical application of immobilized enzymes is likely to be slow because of the special constraints applicable to the use of these materials for human therapy. A possibility for the future is the targetting of immobilized enzymes to particular sites by the attachment of materials capable of binding to specific cell receptor sites.

Progress is being made in the synthesis of molecules that have enzymic properties. At present this work is in its infancy, but examples of enzyme-like

specificity have been demonstrated using modified cyclodextrins (Breslow, 1983). However, at present in only a small number of cases have reaction rates approaching that of enzymes been achieved. Nevertheless, the possibilities for synthesizing artificial enzymes have been demonstrated.

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