Immobilized Enzymes

By C. J. Suckling
DEPARTMENT OF PURE AND APPLIED CHEMISTRY, UNIVERSITY OF STRATHCLYDE, 295 CATHEDRAL STREET, GLASGOW G1 1XL

1 Introduction

Chemists working closely with natural products or biological processes have been aware for a long time of the advantages that the use of enzymes as catalysts would have. Enzymes demand attention chiefly because of the efficiency, selectivity, and mildness with which they catalyse reactions but their use has been restricted because, being high molecular weight proteins, their stability is limited. One possible way out of this dilemma is to use the chemical principles of enzymic catalysis that have been established by physical-organic chemical studies to design stable, synthetic catalysts. It is hoped that such systems will rival enzymes in their efficiency and this approach has been called 'biomimetic chemistry'.[1] An alternative is to invent some way of enhancing the thermal, mechanical, and chemical stability of an enzyme so that it becomes a recoverable catalyst. The chemistry of immobilized enzymes has grown from this germinal idea.

An immobilized enzyme molecule is prevented from diffusing freely through the reaction medium by being attached physically or chemically to a support material: the reaction system thus consists of two phases, the bulk solution, and the immobilized enzyme with its support environment. Commonly the support materials are synthetic polymers and the first indications that such materials might be chemically compatible with biological macromolecules came from Merrifield's pioneering studies on solid-phase peptide synthesis.[2] Shortly afterwards, there developed applications of his techniques to immobilize chemical reagents.[3] In both fields, the range of polymers used was gradually extended from hydrophobic polystyrenes to hydrophilic organic polymers. The development of much of the polymer chemistry required for enzyme immobilization has run parallel with studies of enzyme purification by the technique of affinity chromatography.[4] In addition, enzymes have been immobilized upon inorganic and non-polymeric supports and the wide range of immobilization techniques available opens up a multitude of potential applications.

The chief areas of current application of immobilized enzymes are in clinical

[1] R. Breslow, *Chem. Soc. Rev.*, 1972, **1**, 553.
[2] R. Merrifield and G. R. Marshall, in 'Biochemical Aspects of Reactions on Solid Supports', ed. G. R. Stark, Academic Press, New York, 1971, p. 111; L. J. Marnett, D. G. Neckers, and A. P. Schaap, in 'Techniques in Chemistry', ed. J. B. Jones, C. J. Sih, and D. Perlman, Interscience, New York, 1976, Vol. 10, p. 995.
[3] C. C. Leznoff, *Chem. Soc. Rev.*, 1974, **3**, 65.
[4] H. Guilford, *Chem. Soc. Rev.*, 1973, **2**, 249.

chemistry and in the food processing industry. Clinical chemists need to analyse for metabolites accurately, rapidly, and repeatedly; soluble enzymes are already widely used for this purpose. The industrial conversions of milk into cheese and of corn syrup into fructose are also catalysed by enzymes. If immobilized enzymes were used for these tasks, there would be a considerable saving of materials and money. The potential of applications such as these has stimulated much basic research into the modification of enzyme properties by immobilization and into the design and operation of both analytical and large-scale immobilized enzyme reactor systems. Much has already been written in detail on these topics.[5-8] Rather than compose new variations upon often heard themes, this review will try to show how the chemical properties of immobilized enzymes fit them for their application in general terms.

As is often the case in those areas of chemistry related to biology, nature evolved the chemistry of interest before the chemist invented it. Immobilized enzymes are a good example of this. Many metabolic enzymes are not diffusing freely in solution but are compartmentalized, agglomerated, or bound to membranes. These three natural immobilization techniques are exemplified firstly by the enzymes of the citric acid cycle and of oxidative phosphorylation, which are contained within subcellular particles known as mitochondria, secondly by the fatty-acid-synthesizing enzyme complex, in which a high metabolic efficiency is achieved by a number of enzymes acting in concert, and thirdly by several enzymes that hydroxylate foreign compounds. The last group of enzymes is bound to lipid membranes in liver and it is very difficult to free the enzymes from the lipid without destroying their catalytic activity. The membrane thus provides a safe working environment for the enzyme. Chemists have endeavoured to incorporate each of these features into synthetically immobilized enzyme systems and the following discussion illustrates how far they have succeeded.

2 Techniques of Immobilization

The applications mentioned in the introduction require enzymes immobilized in quite different forms. For instance an analyst might need an enzyme bound to a flexible membrane that he can attach to an electrode; in contrast, an enzyme immobilized upon a rigid support might suit an industrial reactor. In any case, it is obviously essential that the catalytic activity of an enzyme is not seriously impaired by the immobilization process. In this respect, it could be argued that a physical means of immobilization such as adsorption upon a suitable material would be preferable to chemical methods in which the enzyme becomes covalently bonded to its support. However, the regions of enzymes at which catalysis takes place, the so-called active sites, are rarely found on the surface

[5] O. R. Zaborsky, 'Immobilized Enzymes', CRC Press, Cleveland, 1973.
[6] 'Handbook of Enzyme Biotechnology', ed. A. Wiseman, Wiley, New York, 1975.
[7] K. Mosbach, in 'Techniques in Chemistry', ed. J. B. Jones, C. J. Sih, and D. Perlman, Interscience, New York, 1976, Vol. 10, p. 969.
[8] 'Immobilized Enzyme Principles', Applied Biochemistry and Bioengineering, Vol. 1, ed. L. B. Wingard, E. Katchalski-Katzir, and L. Goldstein, Academic Press, New York, 1976.

of the protein molecule; they are usually clefts in the molecule's interior. This means that a bulky reagent such as a polymer can be coupled to the surface of an enzyme molecule without disrupting the catalytic machinery. Provided that extremes of pH and ionic strength, temperatures greater than 40 °C, and small, highly electrophilic reagents are avoided in the immobilization process, both physical and chemical techniques are generally applicable.

A. Physical Immobilization Methods.—One of the simplest techniques for immobilizing an enzyme is to adsorb it upon an inert, insoluble matrix.[9] Many metal oxides, including glasses, will function as matrices for adsorption, principally by hydrogen-bonding between oxygen functions of the oxide surface and polar groups on the enzyme's periphery. Similarly, ion-exchange resins will bind enzymes by ionic association with charged groups on the surface of the enzyme. Recently, hydrophobic association has been used as an immobilization technique.[10] With these procedures it is common to find that some desorption and leakage of the enzyme into the bulk medium occurs and consequently more tenacious immobilization techniques are required.

If a gel is prepared in a solution containing an enzyme, the enzyme becomes trapped within the forming gel matrix. Since the molecular weights of enzymes exceed 15 000, it is easy to prepare a gel with a pore size too small to allow the enzyme to escape. This has been achieved with carbohydrate and polyacrylamide gels, both of which are essentially hydrophilic.[9] If a hydrophobic container for the enzyme is required, the enzyme can be encapsulated within droplets of polymerizing monomers and the resulting microcapsules containing the enzyme can be filtered from the polymerization suspension.[11] Less permanent methods that are on the fringe of immobilization are liquid emulsions[12] and aqueous–organic two-phase systems.[13] Finally, small but highly efficient immobilized enzyme reactors can be prepared by enclosing enzymes within semi-permeable devices; bundles of hollow fibres have been especially successful.[14]

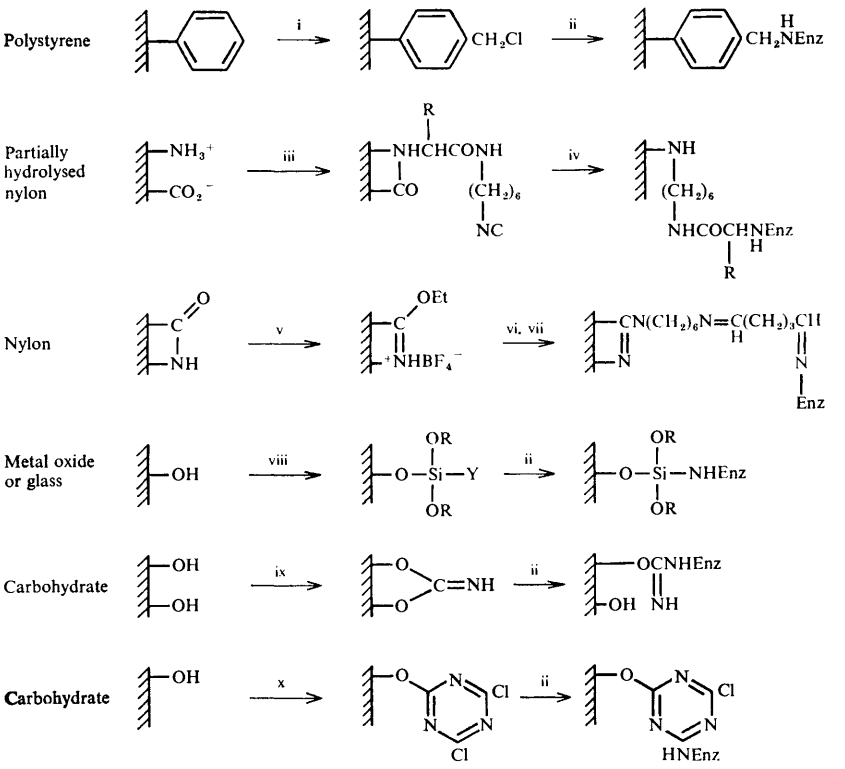
In all of the physical methods of immobilization that enclose an enzyme within a small pored wall the size of the pores controls the transport of molecules to and from the enzyme's active site. This effect may be detectable either as a change in the kinetic properties of an enzyme or, more dramatically, as an alteration in the substrate specificity of the enzyme. Since the chemist can determine the pore size, the opportunity exists to control the reactivity of the enzyme.

B. Chemical Immobilization Methods.—There is usually an abundance of

[9] R. Goldman, L. Goldstein, and E. Katchalski, in 'Biochemical Aspects of Reactions on Solid Supports', ed. G. R. Stark, Academic Press, New York, 1971, pp. 4—5.
[10] K. C. Caldwell, R. Axén, M. Bergwall, and J. Porath, *Biotechnol. Bioeng.*, 1976, **18**, 1573, 1589.
[11] 'Microencapsulation', ed. J. E. Vandegaer, Plenum Press, New York, 1974.
[12] S. W. May and N. N. Li, in 'Enzyme Engineering', ed. E. K. Pye and L. B. Wingard, Plenum Press, New York, 1974, Vol. 2, p. 77.
[13] A. Pollak and G. M. Whitesides, *J. Amer. Chem. Soc.*, 1976, **98**, 289.
[14] P. R. Rony, *J. Amer. Chem. Soc.*, 1972, **94**, 8247.

nucleophilic groups (amino-acid side-chains) on the surface of enzyme molecules. Using these groups as reactants, three distinct types of immobilization are possible.[15]

Firstly, an enzyme can be covalently bonded through a peripheral reactive group to a suitably functionalized polymer (Scheme 1). Polystyrenes are usually



Scheme 1

Reagents: i, $EtOCH_2Cl$; ii, Enzyme-NH_2; iii, RCHO-$CN(CH_2)_6NC$; iv, RCHO-Enzyme-NH_2; v, $Et_3O^+BF_4^-$; vi, $H_2N(CH_2)_6NH_2$; vii, $OCH(CH_2)_3CHO$; viii, $Si(OR)_3Y$ (Y = CN, SH, *etc.*); ix, CNBr; x, cyanuric chloride

activated by preparing chloromethylated derivatives following Merrifield.[2] Metal oxides can be treated with silylating agents bearing reactive groups with which the enzyme can bond.[16] Polyamides, after partial hydrolysis, can be reconstituted incorporating the enzyme into the polymer through amide bonds.[17] This method is suitable for large-scale uses of nylon, but on a smaller laboratory

[15] R. D. Falb, ref. 12, p. 67.
[16] G. Baum and M. Lynne, *Process. Biochem.*, 1975, **10**, No. 3, p. 14.
[17] L. Goldstein, A. Freeman, and M. Sokolovsky, ref. 12, p. 97.

scale the activation of amides by conversion into an imino-ether has been used to prepare an automatic analyser for glucose.[18] Recently, radiolysis of acrylic and alkene polymers has been used to graft enzymes to a support[19] and such immobilized systems are characterized by high resistance to microbial attack. Carbohydrates are usually activated with cyanogen bromide or cyanuric chloride.[20,21] For laboratory work, a number of commercial polymers with pendant reactive groups are available (agaroses from Pharmacia with amino or carboxylate functions, 'Enzacryls' from Koch Light with amino, hydrazino, thiol, or thiolactone functions).

The second group of chemical techniques involves the incorporation of the enzyme by covalent bonding into a growing polymer chain. To obtain water-insoluble products it is usual to employ copolymers such as acrylamide or acrylic acid with maleic anhydride in which a degree of cross-linking occurs.[22] Although stable preparations usually result, this method runs the risk of wasting a quantity of enzyme by confining it within recesses of the polymer that are inaccessible to substrate molecules.

Thirdly, some enzymes can be immobilized by cross-linking the enzyme molecules to each other with polyfunctional reagents such as bis-diazonium salts, dialdehydes, or cyanuric chloride.[23] This technique is not generally applicable because the small cross-linking reagents may penetrate the enzyme's active site and react there, causing inactivation of the enzyme. However, bifunctional reagents are more widely useful in stabilizing physically adsorbed enzymes by cross-linking.[24]

One of the chief drawbacks of all of these chemical methods for large-scale applications is the cost of the support material. Recently it has been suggested that the reversible chemical immobilization of an enzyme might offer a solution to this problem. Once an enzyme has lost its catalytic activity through prolonged use, it could be removed from the valuable support which is then recovered.[25] Enzymes that have free, non-catalytic thiol groups have been successfully used to demonstrate this principle. The thiols can be oxidized to form disulphide bridges with pendant thiols on the support. When it is desired to strip the enzyme from the support, the disulphide bond can be cleaved with a suitable reagent such as another thiol.

Just as physical immobilization techniques impose a barrier to substrates reaching the active site of the enzyme, so chemical methods modify the immediate environment of the enzyme molecule at the same time as restricting

[18] D. L. Morris, J. Campbell, and W. E. Hornby, *Biochem. J.*, 1975, **147**, 593.
[19] J. L. Garnett, R. S. Kenyon, and M. J. Liddy, *J.C.S. Chem. Comm.*, 1974, 735; H. Maeda, H. Suzuki, A. Yamauchi, and A. Sakamane, *Biotechnol. Bioeng.*, 1975, **17**, 119.
[20] H. H. Weetall and C. C. Betar, *Biotechnol. Bioeng.*, 1975, **17**, 295.
[21] G. Kay and M. D. Lilly, *Biochim. Biophys. Acta*, 1970, **198**, 276.
[22] D. Jaworek, ref. 12, p. 105.
[23] R. Goldman, L. Goldstein, and E. Katchalski, in 'Biochemical Aspects of Reactions on Solid Supports,' ed. G. R. Stark, Academic Press, New York, 1971, p. 22.
[24] M. Charles, R. W. Coughlin, E. K. Paruchuci, B. R. Allan, and F. X. Hasselberger, *Biotechnol. Bioeng.*, 1975, 17, 203.
[25] J. Carlsson, R. Axén, and T. Unge, *European J. Biochem.*, 1975, **59**, 567

access to the enzyme. Through both of these mechanisms the chemist gains a measure of control over the enzyme's reactivity and this is a major stimulus for research into the modification of properties that occurs when an enzyme is immobilized.

3 The Effect of Immobilization upon the Properties of the Enzyme

A. Stability.—To achieve a successful application of an immobilized enzyme it is of great importance to know under what conditions the preparation can be stored and used. The immobilization techniques outlined above cause minimal changes in the conformation of the enzyme molecule and consequently any improvements in the apparent robustness of the enzyme will be functions of the protection that its new environment offers. These are, however, only broad generalizations and each case must be treated on its merits.

Storage stability is usually at least as good as for soluble enzymes under the same conditions of pH, ionic strength, and temperature.[26] At best, greater than 60% of initial activity can be retained over several years' storage. It appears that physically immobilized enzymes fare better than chemically treated ones and there are a few examples of stable preparations that have used a combination of physical and chemical techniques. This is most effective when a pre-adsorbed enzyme can be clipped into its active conformation by a cross-linking agent.[24,27]

A wider range of operating conditions can be expected to be available with immobilized enzymes compared with their soluble relatives, including some unusual solvents for enzymes. Polar immobilization matrices can protect enzymes against denaturants such as urea and guanidinium ions and it has also been possible to conduct reactions in 76% ethanol–water solutions with the enzyme trypsin, a peptidase, attached to glass.[28] Most matrices offer some protection against microbial degradation of the bonded enzyme. However, the thermal stability of enzymes is rarely enhanced on immobilization and it is consequently not reasonable to expect an enzyme-catalysed reaction to be accelerated by heat. Enzymes in general have a fairly sharply defined pH for optimal catalytic activity. Immobilization sometimes broadens the optimal range and shifts it to a different pH,[29] but the contrary has also been observed.[30]

B. Kinetic Behaviour and Catalytic Activity.—Apart from stability, the potential user of an immobilized enzyme will wish to know what modifications to the catalytic activity and specificity of the enzyme are likely consequences of immobilization. The investigation of these factors requires a study of the kinetic properties of the immobilized enzyme under the conditions of intended use.

[26] J. J. Marshall and M. K. Rabinowitz, *Biotechnol. Bioeng.*, 1976, **18**, 3, 9.
[27] O. R. Zaborsky, ref. 12, p. 115.
[28] H. H. Weetall and W. P. Wann, *Biotechnol. Bioeng.*, 1976, **18**, 105.
[29] L. Goldstein, M. Pecht, S. Blumberg, D. Atlas, and Y. Levin, *Biochemistry*, 1970, **9**, 2322.
[30] L. Goldstein and E. Katchalski, *Z. analyt. Chem.*, 1968, **243**, 375.

Enzyme kinetics[31] centre upon the Michaelis–Menten equation which relates the rate of an enzyme-catalysed reaction to the substrate concentration, [S], the maximum velocity of the reaction when the enzyme is saturated with substrate, V_{max}, and the Michaelis constant, K_m:

$$\text{Rate} = \frac{V_{max}}{1 + K_m/[S]}.$$

The Michaelis constant is a measure of the affinity of the enzyme for its substrate; a small K_m indicates that the substrate is tightly bound by the enzyme. K_m and V_{max} are characteristic properties of an enzyme and both may be altered by the microenvironment of the enzyme's supporting polymer.

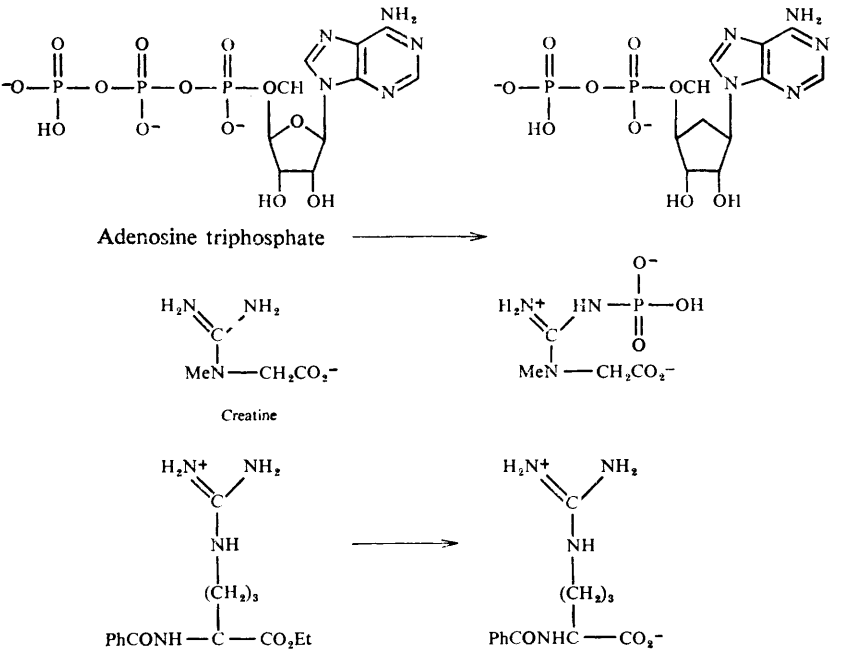
Catalytic activity and specificity are usually measured in terms of relative rates of reactions with different substrates. Many enzymes will tolerate foreign substrates and will transform them into products, usually at rates less than for the natural substrates. Absolute specificity is encountered only with respect to stereoisomeric compounds. Since immobilization can alter the rates at which substrates can react, chiefly by modifying K_m, both the activity and the specificity of an immobilized enzyme may well differ markedly from those of the soluble enzyme. Sufficient is known about the physicochemical factors that affect the Michaelis constant for it to be possible to design an immobilized enzyme, an analytical membrane for instance, with the specificity that the chemist desires.

Once immobilized, an enzyme can be considered to be in a separate phase from the bulk solvent: by making this factorization it is possible to develop a theoretical explanation for the changes in kinetic properties that accompany immobilization.[32] Consider an enzyme immobilized within an anionic matrix, such as produced by carboxylate ions in a maleic anhydride copolymer. Protons in the reaction medium will tend to drift towards the matrix to annul its negative charge. As a result, the enzyme on the matrix will experience a pH lower than the surrounding medium and, in order that the ionizable groups on the enzyme's active site retain their correct ionization state for catalysis, the pH of the bulk phase must be raised to compensate for the 'protonation' of the matrix and the enzyme. This phenomenon is observed as a shift of the pH optimum to higher pH for an enzyme in an anionic environment. A polycationic resin will, of course, shift the pH optimum to lower pH. The pHs prevailing in each domain can be measured and from them an electrostatic potential of 50—150 mV between the two phases can be calculated, a value in good agreement with theoretical calculations.[33]

Related effects can be illustrated quantitatively by measuring the Michaelis constant for the immobilized enzyme. Intuitively it is obvious that K_m will be reduced if the microenvironment due to the support and the substrate are oppositely charged, and *vice versa*. For example, ATP-creatinine phospho-

[31] H. R. Mahler and E. H. Cordes, 'Biological Chemistry', 2nd Edn., Harper and Row, New York, 1971, p. 275.
[32] L. Goldstein, Y. Levin, and E. Katchalski, *Biochemistry*, 1964, **3**, 1913.
[33] R. Goldman, L. Goldstein, and E. Katchalski, ref. 23, p. 34.

transferase catalyses the phosphorylation of creatinine by the polyanionic molecule adenosine triphosphate (ATP; Scheme 2).[34] The non-immobilized



Scheme 2

enzyme has K_m = 6.5 × 10^{-4} mol l^{-1} but the enzyme immobilized on carboxymethyl cellulose, an anionic polymer, had the less strongly bound value of 7 × 10^{-3} mol l^{-1}. In contrast, the peptidase ficin hydrolysed benzoylarginine ethyl ester, a cationic substrate, with K_m = 2 × 10^{-2} mol l^{-1} in the soluble state but K_m = 2 × 10^{-3} mol l^{-1} when immobilized on carboxymethyl cellulose. To emphasize that there is a difference between the kinetic constants for free and immobilized enzyme, the Michaelis constant for the latter is written K_m'.

Since the immobilization of an enzyme upon a charged support introduces an electric field effect into the enzyme's kinetic behaviour, it has been suggested that the application of an external electric field could generate useful properties. A theory of these effects has been propounded.[35]

A further distortion of kinetic parameters is caused by diffusion limitations in immobilized preparations. An insoluble enzyme particle in solution is immediately surrounded by a layer of solvent that is unstirred. As the substrate is consumed

[34] W. E. Hornby, M. D. Lilly, and E. M. Crook, *Biochem. J.*, 1968, **107**, 669.
[35] Y. Seto and S. T. Hsieh, *Biotechnol. Bioeng.*, 1976, **18**, 813.

within the immobilization particle during reaction, a substrate concentration gradient will be set up leaving the immediate environment of the enzyme depleted in substrate with respect to the bulk solution. Consequently more substrate will be required in order to saturate the enzyme's active sites and hence to attain maximal rates of reaction. In other words, K_m' for the immobilized enzyme will be larger than K_m for the soluble enzyme. The obvious expedients for circumventing this difficulty have proved successful. Smaller immobilized particles allow K_m' to approach K_m more nearly,[23] as does increasing the stirring rate of the reactor. In the limit, the best that can be done is to immobilize the enzyme upon a soluble support such as a short-chain polysaccharide in aqueous solution. Thus chymotrypsin, a peptidase, has $K_m = 3.3 \times 10^{-3}$ mol l^{-1} for the non-natural substrate acetyltyrosine ethyl ester; the enzyme immobilized upon a water-insoluble Sephadex showed $K_m' = 3 \times 10^{-2}$ mol l^{-1} whereas an analogous water-soluble derivative had $K_m' = 3 \times 10^{-3}$ mol l^{-1}.[36] Extending this notion, the more soluble the enzyme support in the reaction medium, the more reactive the preparation is likely to be, but at the cost of the loss of the convenience of an insoluble preparation in recovery of the enzyme. A general theory of both diffusion and electrostatic effects on immobilized enzymes has been put forward by Lilly.[34]

4 Applications of Immobilized Enzymes

A characteristic that distinguishes two broad classes of applications of immobilized enzymes is the contribution that further technological research and development has made to the realization of the application. On the one hand, for mechanistic studies of enzyme catalysis, for research into membrane properties, and for laboratory synthesis, a bottle of immobilized enzyme, a beaker, and a stirrer will usually be adequate hardware. On the other hand, industrial, analytical, and medical applications require much research into the whole system that surrounds the immobilized enzyme. It is perhaps as much a reflection of the technological problems to be solved as an indication of the intense current interest in applications of immobilized enzymes that publications of industrial, analytical, or medical significance appear more frequently than those dealing with the more academic aspects of the field.

A. Biochemical and Bio-organic Research.—Immobilization of enzymes opens up new methods for the study of chemical interactions within proteins: interactions between sections of a polypeptide chain give rise to the biologically active forms, the tertiary structure, or folded protein chain, and to the quaternary structure (*i.e.* the agglomeration of completely folded chains or subunits). By immobilization upon an inert support, a protein chain can be prevented from interacting with other proteins in solution and a single subunit can then be studied in complete isolation.

A postulate of protein chemistry is that a given sequence of amino-acids will

[36] R. Axén, P. A. Myrin, and J. C. Janson, *Biopolymers*, 1970, **9**, 401

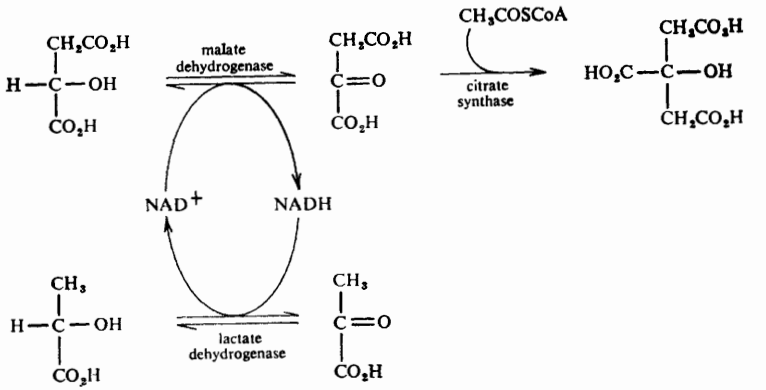
spontaneously fold into a specific tertiary structure which, for an enzyme, will be the catalytically active structure. It has been possible to verify this postulate for simple, single subunit enzymes but the complications caused by subunit agglomeration have made studies of multicomponent enzymes difficult. Lactate dehydrogenase, an enzyme that has four subunits, has been immobilized on glass and it was found that after mild denaturation of the enzyme, causing the tertiary and quaternary structure to be disrupted, activity was recoverable on removal of the denaturing agent.[37] This result confirms that careful immobilization does not interfere with the folding of polypeptide chains, at least when glass is the support.[38]

Aldolase is another tetrameric enzyme and it catalyses the reversible cleavage of fructose 1,6-diphosphate into dihydroxyacetone phosphate and glyceraldehyde 3-phosphate. By means of Sepharose activated to only a small degree, it was possible to immobilize the whole enzyme globule intact and subsequently to split off most of the subunits, leaving immobilized only those few single polypeptide chains that were covalently bonded to the support. The polymer held these immobilized subunits sufficiently far away from each other to prevent interaction between them, and the properties of the individual subunits could then be studied. Interestingly the single subunits showed some enzymic activity and, as with lactate dehydrogenase, reconstitution of the tetrameric enzyme restored almost full catalytic activity.[39]

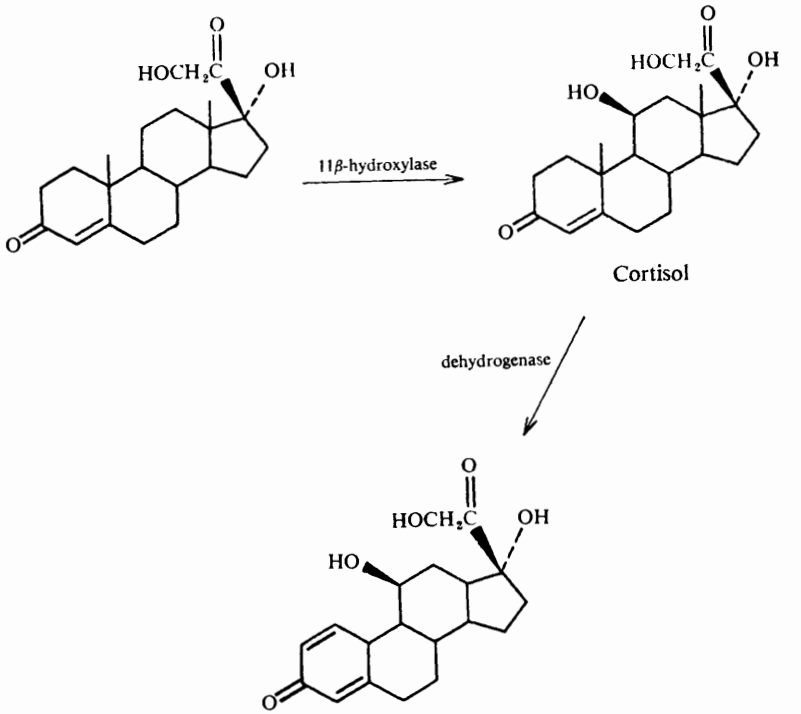
In the Introduction it was pointed out that many functional units in biology consist of groups of enzymes embedded in membranes, one example being the mitochondrial enzymes of the tricarboxylic acid cycle. To understand the behaviour of such enzyme systems it is valuable to investigate the ways in which smaller groups of enzymes function when acting in concert. Synthetically immobilized enzymes offer an opportunity to study such systems, and Mosbach has immobilized malate dehydrogenase, citrate synthase, and lactate dehydrogenase together and studied their behaviour as a model for oxalacetate production in mitochondria.[40] His immobilized trio was eight times more active than the three soluble enzymes interacting randomly in solution (Scheme 3).

B. Synthetic Organic Chemistry.—Enzymes have potential as catalysts for synthetic operations but, biosynthetic studies apart, little has yet been achieved.[41] In natural product chemistry it may be expected that an immobilized enzyme will accept many substrates structurally related to its natural substrates. With steroids especially, enzymes from micro-organisms have wide specificities and it has been possible to use columns of immobilized fungal cells having 11-β-hydroxylase and 1,2-dehydrogenase activity in syntheses of cortisol and prednisolone (Scheme 4).[42]

[37] H. E. Swaisgood and I. C. Cho, *Biochim. Biophys. Acta*, 1972, **258**, 675.
[38] H. R. Horton and H. E. Swaisgood, ref. 12, p. 169.
[39] W. W.-C. Chan, *Biochem. Biophys. Res. Comm.*, 1970, **41**, 1198.
[40] P. A. Srere, B. Mattiason, and K. Mosbach, *Proc. Nat. Acad. Sci., U.S.A.*, 1973, **70**, 2534.
[41] C. J. Suckling and K. E. Suckling, *Chem. Soc. Rev.*, 1974, **3**, 387.
[42] K. Mosbach and P. M. Larsson, *Biotechnol. Bioeng.*, 1970, **12**, 19.



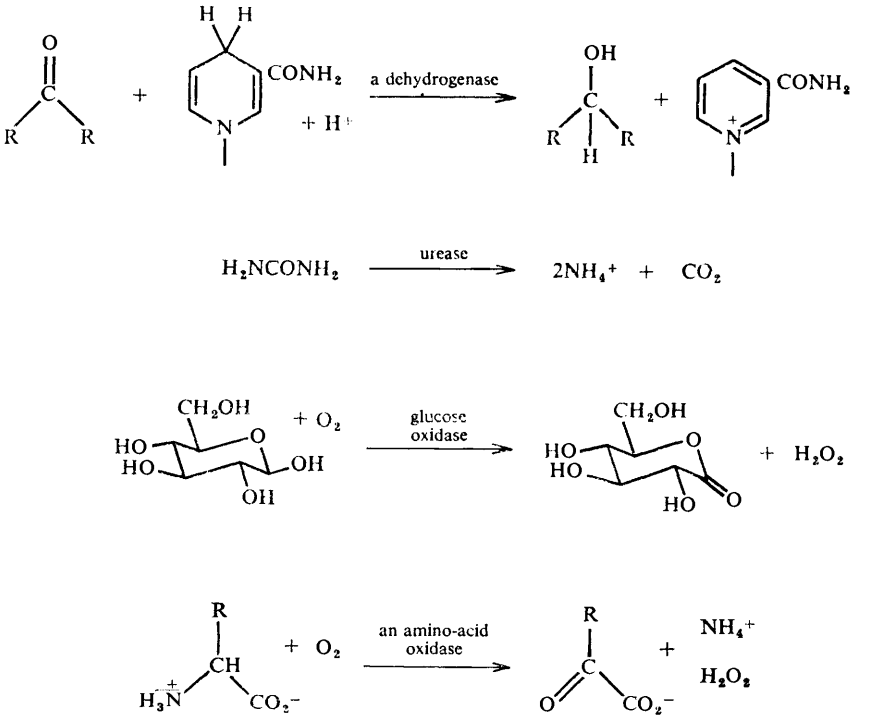
Scheme 3



Scheme 4

Many enzymes of synthetic potential, such as alcohol dehydrogenase, require coenzymes in addition to substrate in order to function. It is often difficult to regenerate the consumed coenzyme with chemical reagents and immobilized enzymes thus become obvious choices for the task. The problem of regenerating the redox coenzyme nicotinamide adenine dinucleotide has been solved for small-scale work but remains a difficulty when larger-scale operations are contemplated. However, the synthesis of adenosine triphosphate from adenosine monophopshate and chemically prepared acetyl phosphate by means of a two-enzyme coupled system immobilized on Sephadex or polyacrylamide has been successfully developed to the verge of being a practical process.[43]

C. Analytical Applications.—Enzymes have for many years been used in the routine assay of metabolites in biological fluids but each analysis necessarily consumes a quantity of soluble enzyme. An obvious improvement is to employ a re-usable immobilized enzyme preparation. Compounds such as glucose, lactic acid, urea, and ethanol are particularly amenable to this approach because suitable specific enzymes are readily available (Scheme 5). The concentrations of



Scheme 5

[43] C. R. Gardner, C. K. Colton, R. S. Langer, B. K. Hamilton, M. C. Archer, and G. M. Whitesides, ref. 12, p. 209; G. M. Whitesides, A. Chmurny, P. Garrett, A. Lamotte, and C. K. Colton, *ibid.*, p. 217.

metabolites present in the sample are determined by measurement of the change in concentration of either a co-reactant, such as NADH in dehydrogenase-catalysed reactions,[44] or a product such as amonium ions from the hydrolysis of urea by urease.[45] Spectrophotometry and potentiometry are the respective analytical techniques. However, for those enzymes that consume oxygen as a reactant, the oxygen concentration is best followed by means of an oxygen electrode[46] and it is a short step to combine the enzyme with the electrode membrane to produce the so-called enzyme electrode.[45,46]

An enzyme electrode consists of a glass electrode surrounded by a membrane that contains the immobilized enzyme. It is sometimes sufficient simply to parcel the enzyme up between the glass and a semipermeable membrane such as cellophane, but more usually the enzyme is covalently bound to the membrane. With such a system, three modes of analytical selectivity are available. Firstly, there is the enzyme's intrinsic selectivity, secondly, the glass electrode can be chosen to respond only to certain ions,[47] and thirdly, the choice of the membrane (anionic, cationic, hydrophobic, *etc.*) offers selectivity. Many applications of enzyme electrodes are built into automated systems, *e.g.* glucose oxidase with an oxygen electrode.[46] Similarly, amino-acid oxidases, which catalyse the oxidative deamination of amino-acids to keto-acids (Scheme 5) can be used in conjunction with a cation-selective electrode to permit rapid and continuous assay for amino-acids.[48] A more detailed discussion of these techniques, including multienzyme assays can be found in a review by Guilbalt.[49]

Automated amino-acid analysis is a well established technique and immobilized enzymes make it possible to extend automation to the peptide degradation processes. In peptide analysis, the chief advantage of enzyme-catalysed degradation is that amino-acid side-chains such as the alcohol functions of serine and threonine and the indole ring of tryptophan, which are decomposed by the normal acidic hydrolysis conditions, are quite stable to enzymes. Studies of immobilized derivatives of the broad-spectrum peptidase, pronase and of other more selective peptidases show that enzymic degradation has the potential for development into fully automatic analysers.[50] Research has also begun into the use of immobilized phosphatases for the hydrolysis and sequencing of nucleic acids.[51]

It should not be thought that the scope of analysis using immobilized enzymes is limited to organic compounds. Hydrogen peroxide is a product of some of the oxidation reactions in Scheme 5 and it can readily be determined using immobilized peroxidase.[52] Further, some enzymes have obligatory requirements

[44] M. K. Weibel, ref. 12, p. 385.
[45] G. G. Guilbalt and J. G. Montalvo, *J. Amer. Chem. Soc.*, 1969, **91**, 2164.
[46] S. J. Updike and G. P. Hicks, *Science*, 1967, **158**, 270; *Nature*, 1967, **214**, 986.
[47] R. A. Durst, *Amer. Scientist*, 1971, **59**, 353.
[48] G. G. Guilbalt and E. Hrabankova, *Anal. Chim. Acta*, 1971, **56**, 285.
[49] G. G. Guilbalt, *Enzymology*, 1975, **1**, 293.
[50] G. P. Royer and G. M. Green, *Biochem. Biophys. Res. Comm.*, 1971, **44**, 426.
[51] R. A. Zingaro and M. Uziel, *Biochim. Biophys. Acta*, 1970, **213**, 371.
[52] B. F. Rocks, *Proc. Soc. Analyt. Chem.*, 1973, **10**, 164.

for certain metal ions. Tyrosinase, which catalyses phenol oxidation, requires copper(II) ions and these ions can be removed from the immobilized enzyme which becomes inactive. If the enzyme is then immersed in a solution containing an unknown concentration of copper, the extent of reactivation of the enzyme gives a measure of the copper concentration. Nanogram quantities are reported to be detectable.[53] An assay for zinc can be set up similarly using immobilized alkaline phosphatase.[54]

A recent development that is causing much interest in the biomedical field is the use of immobilized enzymes in immunoassay techniques as an adjunct to radioimmunoassay.[55] An antibody to an enzyme can be rapidly purified by means of an immobilized enzyme: if the system is then reversed and the purified antibody itself is immobilized, a material that will absorb the target enzyme with high selectivity is produced and this facilitates the determination of the concentration of the enzyme in a sample.

In this survey of analytical methods, little has been said about the type of immobilization technique best suited to the application. Much analytical work requires enzyme membranes and, accordingly, either covalent attachment to or entrainment within a polymer is suitable. On the other hand, a continuous analyser involving degradative enzymes would need enzymes adsorbed or bonded to materials suitable for use in columns such as glasses or polymer beads. The variety of approaches being pursued is well illustrated by contributions to the 1973 Symposium on Enzyme Engineering.[56]

D. Medical Applications.—The idea of using enzymes as drugs is not new; protein- and carbohydrate-hydrolysing enzymes, for instance, are widely used as digestive aids, especially in Japan. However, the use of immobilized enzymes in medicine has wider therapeutic implications which can be regarded chiefly as either the removal of toxic substances or the synthesis of metabolites that are in deficiency.[57,58] It has been suggested, for example, that urease could be used to control urea levels and that phenylalanine ammonia lyase could help alleviate the problem of phenylketonuria.

The attraction of using an immobilized enzyme in place of a soluble one in medicine is four-fold. Firstly, hydrolysis of an enzyme drug by digestive processes can be avoided; secondly, the elimination of the enzyme by filtration through the kidneys is prevented; thirdly, immunological rejection of the enzyme drug can be controlled; and finally, the drug can be localized into its required site of action. These considerations apply to the use of immobilized enzymes in invasive therapy, and clinicians are rightly slow to adopt new invasive methods

[53] J. V. Stone and A. Townshend, *J.C.S. Chem. Comm.*, 1972, 502.
[54] A. Townshend and A. Vaughan, *Talanta*, 1970, **17**, 289.
[55] D. L. Eshenbaugh and E. James, in 'Immobilized Biochemicals and Affinity Chromatography', ed. R. B. Dunlap, Plenum Press, New York, 1974, p. 61.
[56] 'Enzyme Engineering', ed. E. K. Pye and L. B. Wingard, Plenum Press, New York, Vol. 2, 1974.
[57] G. Brown, ref. 12, p. 433.
[58] T. M. S. Chang, *Enzymology*, 1975, **1**, 245.

until both their efficiency and safety have been rigorously established. There are also practical difficulties to surmount. The effectiveness of any treatment using an immobilized enzyme will depend upon the ability of the required substrates and cofactors to reach the enzyme and upon the active lifetime of the enzyme. Solutions to these problems have still to be found but the following example illustrates what may become possible in the future.

The control of urea levels in blood has been studied by Chang using immobilized preparations of urease.[59] Both the implantation of immobilized enzyme particles and the construction of extracorporeal shunt devices have been investigated. Urease is especially suitable for pioneering studies because it catalyses a simple hydrolysis reaction without coenzyme requirements. It has been estimated that an artificial kidney as small as 10 cm long × 2 cm diameter is feasible using these techniques.

Apart from the treatment of patients, immobilized enzymes have been shown to be useful in clearing air in rooms from virus particles. In order to reproduce in the host and cause disease, viruses depend upon the translation of their nucleic acids by the infected host cells. If enzymes that specifically hydrolyse the viral nucleic acids can be immobilized, the virus will be killed upon contact with the enzyme. Porous glass and ceramic materials have been used as supports for such enzymes and the materials have been shown to disinfect airborne herpes simplex, influenza, and cocksackie viruses rapidly.[60]

E. **Industrial Applications.**—The idea of using enzymes in large-scale processes goes back to the early twentieth century when Röhm introduced hydrolytic enzymes into laundering.[61] Today, a wide range of processes falls within the scope of industrial applications of enzymes, but, whereas applications to research, analysis, and medicine require only small amounts of enzyme that can easily be obtained from small batches of a natural source, a large-tonnage application would necessitate a substantial investment in the production of the enzyme itself. In addition there are toxicological problems associated with the large quantities of biological waste that would be produced[62] and there is also the high cost of enzyme immobilization itself, with respect to both the support material and the immobilization reagent. Together these factors make it improbable that any of the large-tonnage petrochemical plants, which are highly efficient, will be superseded by plants based upon enzyme technology in the immediate future. However, in those industries where natural products are synthesized either for food or for pharmaceutical purposes, the application of immobilized enzymes is now having a major impact.

(*i*) *Reactor Design.* Once the immobilization and isolation problems are solved, there remains the technological problem of the form that the catalyst should

[59] T. M. S. Chang, *Sci. J.*, 1967, **3**, 62; T. M. S. Chang, L. J. Johnson, and O. J. Ransome, *Canad. J. Physiol. Pharmacol.*, 1967, **45**, 705.
[60] J. Enright, J. Gainer, and D. J. Kirwan, *Environ. Sci. Technol.*, 1975, **9**, 586.
[61] O. Röhm, Ger. P. 283 923.
[62] L. D. Scheel, D. E. Richards, V. B. Perone, and W. B. Tolos, ref. 12, p. 351.

take and the type of reactor that is appropriate.[63-66] Two chief factors must be considered. Firstly, the enzyme must be readily removable from the reaction mixture and, secondly, the kinetics of the immobilized enzyme system must be well established so that the optimum contact time for the reactants can be defined. As we shall see, these factors interact.

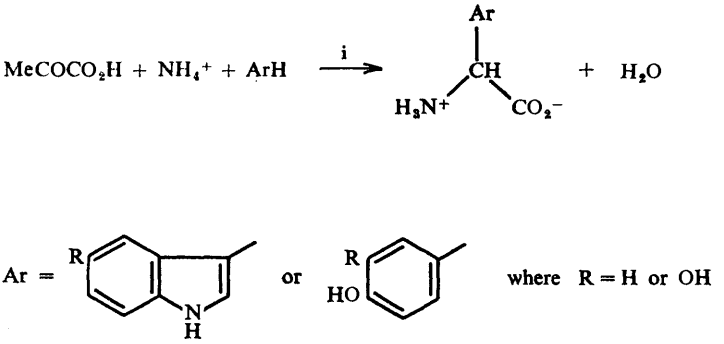
Ready removal of the enzyme can be simply accomplished by packing a column with the particles bearing the enzyme and passing a solution of the reactants through at a rate such that only products are eluted from the column. To determine the correct flow rate, the kinetic parameters for the immobilized enzyme system must be known. It has been deduced theoretically for a column reactor that the conversion into product depends upon the height of and the flow rate through the column, provided that the enzyme is saturated by an excess of substrate. The saturation concentration of substrate depends upon K_m', and it has been shown that the theoretical principles are born out well by experiments with immobilized hydrolytic enzymes.[67-69] The flow rate will further be influenced by the material upon which the enzyme is immobilized. For this reason, rigid supports such as metal oxides are often preferred to organic polymers, provided that stable bonding between the enzyme and the metal oxide can be obtained.

Apart from columns, fluidized-bed, packed-bed, and stirred-tank reactors have been investigated for use with enzymes,[70] the choice usually depending on the reaction in question. In stirred reactors, K_m' varies with the degree of agitation, indicating the importance of substrate diffusion effects as was noted earlier. Many enzyme-catalysed reactions are slow compared with conventional chemical processes and in these cases batch or plug flow processes are preferable. Organic supports have not been notably successful in such reactors because a sufficiently high degree of agitation to overcome diffusion limitations cannot be attained. Consequently there has been a widespread interest in the use of oxides of ferromagnetic metals as supports; the reactants can then be easily separated from the support by restraining the latter in a magnetic field. So far, these ideas have not been developed beyond initial research experiments.[71,72]

(*ii*) *Processes in Operation.* The Japanese are the undoubted leaders in this field and the production of optically pure amino-acids by immobilized enzyme-catalysed hydrolysis of their acylated derivatives has been an established process in Japan for many years.[73] The reported capacity of these processes is substantial,

[63] H. H. Weetall, ref. 55, p. 191.
[64] W. H. Pitcher, jun. and N. B. Havewala, *Enzymology*, 1975, **1**, 83.
[65] M. D. Lilly and P. Dunnill, *Process. Biochem.*, 1971, **6**, No. 8, p. 29.
[66] A. M. Filbert and W. H. Pitcher, *Process. Biochem.*, 1976, **11**, No. 7, p. 3.
[67] R. Goldman, L. Goldstein, and E. Katchalski, ref. 23, p. 39.
[68] A. BarEli and E. Katchalski, *J. Biol. Chem.*, 1963, **238**, 1690.
[69] M. D. Lilly, W. E. Hornby, and E. M. Crook, *Biochem. J.*, 1966, **100**, 718.
[70] M. D. Lilly, G. Kay, A. K. Sharp, and R. J. H. Wilson, *Biochem. J.*, 1968, **107**, 5p.
[71] M. Charles, R. W. Coughlin, B. R. Allen, E. K. Paruchi, and F. X. Hasselberger, ref. 55, p. 213.
[72] G. Gellf and J. Boudrant, *Biochim. Biophys. Acta*, 1974, **334**, 467.
[73] T. Tosa, T. Mori, N. Fuse, and I. Chibata, *Biotechnol. Bioeng.*, 1967, **9**, 603.

greater than 700 kg day^{-1}.[74] Indeed amino-acid production from cheap feedstocks is a continuing area of interest for immobilized-enzyme technologists. For example, a large number of amino-acids can be prepared by enzyme-catalysed transaminations in the presence of the coenzyme pyridoxal phosphate (Scheme 6). Tryptophan and tyrosine have been manufactured by means of an



Scheme 6

Reagent: i, tyrosine phenol lyase–pyridoxal phosphate

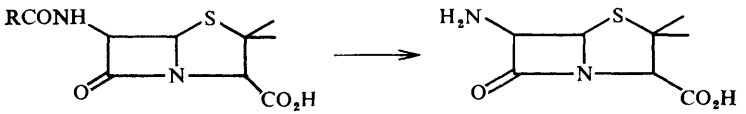
aryl lyase enzyme immobilized on columns from pyruvic acid, ammonia, and either indole or phenol as starting materials.[75] The process also works for hydroxy-substituted indoles and phenols to afford the pharmaceutically important compounds L-DOPA and serotonin. Under optimum conditions, 90% of aromatic substrate is converted into product. The neutral support used, Sepharose, scarcely alters the kinetic properties of the enzyme although the pH optimum is slightly higher for the immobilized system. The chief expense in such a system is the coenzyme, which here was used as a component of the reaction buffer solution.

As we have seen, the problem of coenzyme recovery and regeneration is a severe current limitation of immobilized-enzyme technology, especially where the redox coenzyme, NAD, or the phosphorylating coenzyme, ATP, is involved. The problems of ATP regeneration are being tackled successfully using coupled enzyme systems (see Section 4B) and much effort is currently being expended to find a similar system for NAD.[76,77] Without a solution to this problem, the large-scale use of enzymes catalysing such reactions as the oxidation and reduction of aldehydes, ketones, and alcohols cannot become practicable although the enzymes are readily available.

An interesting immobilized adaptation of established enzyme-catalysed reactions in the pharmaceutical industry is the production of 6-aminopenicillanic

[74] T. Sato, T. Mori, T. Tosa, and I. Chibata, *Arch. Biochem. Biophys.*, 1971, **147**, 788.
[75] S. Fukui and S. Ikeda, *Process. Biochem.*, 1975, **10**, No. 6, p. 3.
[76] R. W. Coughlin, M. Aisiwa, and M. Charles, *Biotechnol. Bioeng.*, 1975, **17**, 209; R. W. Coughlin, M. Aiziwa, M. Charles, and B. F. Alexander, *ibid.*, p. 515.
[77] J. R. Wykes, P. Dunnill, and M. D. Lilly, *Biotechnol. Bioeng.*, 1975, **17**, 51; R. P. Chambers, J. R. Ford, J. H. Allender, and W. Cohen, ref. 12, p. 195.

acid, an important intermediate in the production of semi-synthetic penicillins (Scheme 7).[78] The process uses either carbohydrate or maleic anhydride–methyl vinyl ether polymers and yields of over 90% are claimed in a continuous process. Similar results have been described for the related antibiotics the cephalosporins.[79]



Scheme 7

The food processing industry is a further major area of application of immobilized-enzyme technology; crude enzymes have been used in beer, bread, and cheese production for many years.[80,81] A case that hit the chemical headlines recently was the establishment of a plant to isomerize the cheap but tasteless sugar glucose into its toothsome isomer fructose. The catalyst consists of compounded particles of the whole bacterium that contains the enzyme glucose isomerase, and a column reactor is used. In this way, the expensive isolation and immobilization steps are replaced by the cheaper and simpler operations of growing and harvesting the micro-organism and compressing it into catalyst pellets.[82] The process is attractive because its feedstock, corn syrup, is currently cheap. For those interested in the development of glucose isomerase technology from enzyme isolation to reactor design, an interesting case study has been published.[83]

Cheese making has traditionally used rennet, an enzyme preparation from the stomach mucosa of unweaned calves, to coagulate the milk, but a rise in cheese production has brought about a world shortage of rennet.[84] If an immobilized enzyme that catalyses the same reactions can be obtained from a cheap microbial source and immobilized, the establishment of an economic process seems probable.[85] Development work has shown that milk can be coagulated continuously in a fluidized-bed reactor[86] and it has been estimated that immobilized enzymes could offer up to an eighty-fold saving in the cost of rennins.[87] Dairy processing is likely to prove to be a rapid growth area of enzyme technology.

[78] M. A. Cawthorne, Ger. P. 2 356 630/1974 (*Chem. Abs.*, 1975, **82**, 15 244).
[79] T. Fuji, K. Matsumoto, and T. Watanake, *Process. Biochem.*, 1976, **11**, No. 8, p. 21.
[80] 'Immobilized Enzymes in Food and Microbial Processes', ed. A. C. Olson and C. L. Cooney, Plenum Press, New York, 1973.
[81] H. H. Weetall, *Process. Biochem.*, 1975, **10**, No. 6, p. 3.
[82] J. L. Meers, *Chem. in Britain*, 1976, 115.
[83] B. K. Hamilton, C. K. Colton, and C. L. Cooney, ref. 80, p. 85.
[84] J. L. Sardinas, *Process. Biochem.*, 1976, **11**, No. 4, p. 10.
[85] T. F. Richardson and N. F. Olson, ref. 80, p. 19.
[86] M. Cheryan, P. J. van Wyk, N. F. Olson, and T. F. Richardson, *Biotechnol. Bioeng.*, 1975, **17**, 585.
[87] B. Wolnak, ref. 12, p. 369.

Chemists are also pursuing the long-term potential application of immobilized enzymes to the production of food from metabolic wastes.[88]

5 Conclusions

The market for enzymes used for all purposes is growing continuously and in the U.S.A. in 1975 reached a cash value of £25 million.[87] Very little of this turnover is accounted for by large-scale processes and most industrialists concerned with enzyme technology recognize the market potential of immobilized enzymes. However, they contrast the advantages of mild reaction conditions and selectivity with the drawback of the cost of immobilization. Costs may be cut, as in the glucose isomerase case, by using immobilized whole cells of bacteria and this technique may well prove to have wider applications to reactions in heavy organic chemical industry.

Pye,[89] in a thought-provoking article, is not confident about future developments in industry, partly because of the coenzyme problem that has been discussed. In a more optimistic vein, Mosbach[7] looks forward to the solution of this problem through coupled multienzyme processes and anticipates an era of 'biochemical synthesis' analogous to the era of natural product synthesis that has absorbed so much of the efforts of organic chemists over the years. Undoubtedly, new applications of immobilized enzymes will be developed for analytical and medical purposes. However, major developments in the capital intensive areas of chemistry depend much upon forecasts of the economic situation twenty years from now, in particular in assessing what processes will become too costly to run. Even in special reports on biochemical engineering, little attention has been paid to the contribution that enzymes could make to energy production.[90] While oil is available, immobilized-enzyme technology will not compete with established processes but its use to bridge a future resources gap is a possibility worthy of serious consideration.

[88] D. L. Marshall, ref. 55, p. 345.
[89] E. K. Pye, ref. 80, p. 1.
[90] A. N. Emery, 'Biochemical Engineering Survey', Science Research Council, 1976; E. M. Crook, *Trends in Biochemical Sciences*, 1976, **1**, N.195.