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The Application of Coenzyme-Dependent Enzymes in Biotechnology [and Discussion]

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The application of coenzyme-dependent enzymes in biotechnology

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The principal problems associated with the application of immobilized coenzyme-dependent enzymes in biotechnology are discussed. Particular emphasis is laid on the problems encountered in the covalent immobilization of the nicotinamide nucleotide oxidoreductases and on the special problems posed by the freely dissociable coenzyme. Thus the influence of the immobilization régime on the specific activity and stability of such enzymes and the techniques available for the immobilization, retention and regeneration of the coenzyme moiety are discussed. The solution of the dual problem of retention and regeneration by co-immobilized enzyme–coenzyme systems and the applications of enzyme–coenzyme systems in industry, medicine and analysis are also given. Finally, this report speculates on the future prospects for enzyme–coenzyme systems in biotechnology, how some of the problems may be resolved and how, in some cases, quasi-biological or non-biological systems may represent useful alternatives.

Introduction

Man has enlisted the services of enzymes for many centuries, albeit unwittingly, in the production of cheese, bread and alcoholic beverages. In recent times, however, as the fundamental science of enzymology has unfolded, the colossal potential of enzymes as specific catalysts in industrial manufacturing processes has been recognized (Mosbach 1976; Dunnill 1980; Samejima *et al.* 1980). But despite the fact that enzymes are capable of catalysing complex and specific reactions, efficiently and under mild conditions, their high cost of extraction and purification and their inherent lability has so far severely restricted their penetration into industrial practice. Not surprisingly, therefore, attempts have been made to find cheaper sources and extraction methods for enzymes (Heden 1977) as well as techniques for stabilizing their activity under specified operational conditions (Wiseman 1977; Klibanov 1979; Schmid 1979). Of those enzymes currently being exploited industrially almost all are hydrolases because of their ready availability at low cost and their relative simplicity of action. Enzymes such as carbohydrases, proteases, amidases, lipases and aminoacylases are widely used in the food and pharmaceutical industries for sugar and sugar syrup manufacture, the clarification of beverages, cheese production and milk treatment, the hydrolysis of cellulose, lipids and proteins and for the production of nucleotides, steroids and L-amino acids (Samejima *et al.* 1980). With the advent of immobilized enzyme technology many of these enzymes have been entrapped in, or adsorbed or covalently attached to, solid support matrices and subsequently operated in suitable reactor configurations (Aunstrup 1974; Mosbach 1976). However, the hydrolases are unique among the six main groups of the 2100 or more enzymes recognized by the International Union of Biochemistry in that they do not require low molecular mass cofactors or coenzymes for catalytic activity. The successful application of a host of other enzymes, including oxidoreductases, transferases, lyases, isomerases and ligases, presents more formidable problems because they

require the simultaneous action of non-proteinaceous coenzymes as cosubstrates in the catalytic reactions (Lowe 1979*a*, 1981).

Coenzyme-requiring enzymes

Approximately 40 % of all known enzymes require coenzymes for catalytic activity. For example, dehydrogenases require freely dissociable electron acceptors such as NAD^+ or $NADP^+$ to achieve catalytic oxidoreduction, and many of the phosphotransferases and ligases that catalyse biosynthetic reactions exploit a nucleoside triphosphate such as ATP as coenzyme. Cofactor-requiring enzymic processes are of considerable interest in the transformation of glucose into a variety of chemicals, the conversion of lignin by-products to amino acids, the capture of solar energy by photolysis, the enzymic synthesis of pharmaceuticals such as bacitracin, the epoxidation of fatty acids, the elimination of the beany flavour from soybean products, and in stereospecific steroid, drug and alkaloid transformations (Lowe 1981). Indeed, chemists have long considered the catalytic properties of enzymes as a powerful means for the synthesis of molecules in a regiospecific or stereospecific way. Thus stereospecific oxidations of carbinols to carbonyls can be effected on substrates ranging in complexity from simple aliphatic alcohols to polycyclic alcohols and steroids by using alcohol dehydrogenases of appropriate structural specificities (Jones 1980). Furthermore, one of the main reasons for the use of biological systems in organic synthesis is to create chiral centres by stereospecific hydrogenation of prochiral systems (Tischer *et al.* 1980). Unfortunately, redox reactions of this type require the simultaneous involvement of the complementary coenzyme, in this case the nicotinamide nucleotides NAD^+ and NADH. Since freely dissociable coenzymes of this type are cosubstrates in the catalytic reaction they become exhausted and must be regenerated before reuse. Thus the application of immobilized coenzyme-requiring enzymes depends not only on the inherent idiosyncrasies of enzyme immobilization but also to a large extent on the feasibility of regenerating and reusing the coenzymes in the enzyme reactor (Bright 1975; Mosbach *et al.* 1976; Lowe 1979*a*; Wang & King 1979; Wandrey 1979; Lowe 1981). In this report, particular emphasis will be placed on the immobilization of coenzyme-dependent enzymes such as the nicotinamide nucleotide-dependent oxidoreductases, although much of what is said is equally applicable to ATP-dependent and coenzyme A-dependent enzyme systems (Lowe 1981).

Immobilized oxidoreductases

It has been tacitly assumed that the rules of enzyme immobilization derived mainly from studies on the covalent attachment of relatively low molecular mass monomeric hydrolases such as ribonuclease, α-chymotrypsin and trypsin will apply equally well to the mechanistically more complex multimeric coenzyme-dependent enzymes. Preliminary evidence suggests that this assertion may not necessarily hold because the immobilization and stabilization of such oligomeric enzymes with good retention of catalytic activity has so far met with only limited success. Table 1 lists some nicotinamide nucleotide-dependent oxidoreductases that have been immobilized on solid support matrices. Examination of the examples cited in this table reveals several important points. Firstly, direct comparisons between immobilized enzyme preparations are difficult to make in view of the different sources of enzymes, initial specific activities, immobilization chemistries, loadings, matrix compositions and coupling conditions used by different authors. In most cases the reports contain no quantitative information at all despite

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the recommendations of the Nomenclature Committee on Immobilized Enzymes (Sundaram & Pye 1974). Nevertheless, closer inspection of the examples quoted in table 1 reveals that some immobilized oxidoreductases, notably enoate reductase (Tischer *et al.* 1980), D-glucose 6-phosphate dehydrogenase (Smith & Lenhoff 1974), L-glutamate dehydrogenase (Cooper & Gelbard 1981) and 20β-hydroxysteroid dehydrogenase (Pasta *et al.* 1980; Carrea *et al.* 1982),

TABLE 1. IMMOBILIZED NICOTINAMIDE NUCLEOTIDE OXIDOREDUCTASES

oxidoreductase	EC no.	references
alcohol dehydrogenase	1.1.1.1	Brougham & Johnson (1978), Carrea *et al.* (1982), Hornby *et al.* (1972) Johnson & Brougham (1981), Kelly *et al.* (1977), Koch-Schmidt & Mosbach (1977), Legoy *et al.* (1978, 1980), Manecke & Günzel (1962), Melrose (1971), Millis & Wingard (1981), Ooshima *et al.* (1981), Saronio *et al.* (1974)
enoate reductase		Tischer *et al.* (1980)
formate dehydrogenase	1.2.2.2	Tischer *et al.* (1980), Wichmann *et al.* (1981)
galactose dehydrogenase	1.1.1.48	Sundaram (1977)
glucose dehydrogenase	1.1.1.47	Bisse & Vonderschmitt (1978), Pfeiffer *et al.* (1980)
glucose 6-phosphate dehydrogenase	1.1.1.49	Morris *et al.* (1975), Smith & Lenhoff (1974)
glutamate dehydrogenase	1.4.1.3	Cooper & Gelbard (1981), Havekes *et al.* (1974)
glyceraldehyde 3-phosphate dehydrogenase	1.2.1.12	Carrea *et al.* (1982)
3α-hydroxysteroid dehydrogenase	1.1.1.50	Bovara *et al.* (1981)
20β-hydroxysteroid dehydrogenase	1.1.1.53	Carrea *et al.* (1982), Pasta *et al.* (1980),
Lactate dehydrogenase	1.1.1.27	Chan & Mosbach (1976), Cho & Swaisgood (1974), Dixon *et al.* (1973), Focher *et al.* (1973), Hornby *et al.* (1972), Koch-Schmidt & Mosbach (1977), Kovalenko *et al.* (1981), Wilson *et al.* (1968)
leucine dehydrogenase	1.4.1.9	Wichmann *et al.* (1981)
lipoamide dehydrogenase	1.6.4.3	Lowe (1977*a–c*)
malate dehydrogenase	1.1.1.37	Hornby *et al.* (1972), Mosbach (1980)

display specific activities of 25–80 % relative to the soluble unmodified enzymes. In other cases relative specific activities are recorded as 'low' for D-glyceraldehyde 3-phosphate dehydrogenase (Carrea *et al.* 1982), 14–20 % for L-lactate dehydrogenase (Chan & Mosbach 1976; Kovalenko *et al.* 1981) and 10 % or less for alcohol dehydrogenase (Saronio *et al.* 1974; Brougham & Johnson 1978; Millis & Wingard 1981), L-lactate dehydrogenase (Focher *et al.* 1973) and DL-lipoamide dehydrogenase (Lowe 1977*c*). To a first approximation, it appears that native enzymes that display high initial specific activities (more than 500 μmol substrate transformed min^{-1} mg^{-1}), such as yeast alcohol dehydrogenase (Saronio *et al.* 1974; Millis & Wingard 1981; Ooshima *et al.* 1981), L-malate dehydrogenase (Lowe, unpublished observations) and L-lactate dehydrogenase (Focher *et al.* 1973), are subject to severe losses on immobilization compared with those enzymes such as enoate reductase (Tischer *et al.* 1980), horse liver alcohol dehydrogenase (Koch-Schmidt & Mosbach 1977*a*) and the hydroxysteroid dehydrogenases (Pasta *et al.* 1980; Bovara *et al.* 1981; Carrea *et al.* 1982), where the native specific activities are considerably lower (less than 10 μmol substrate transformed min^{-1} mg^{-1}). Secondly, where apparent K_m

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values for coenzyme and cosubstrate are quoted for the immobilized oxidoreductase listed in table 1, it is clear that the apparent K_m for cosubstrates varies from considerably less to considerably more than the true K_m for the native enzyme. It is likely that these differences in apparent K_m are attributable either to conformational changes in the bound enzyme or to partitioning of the cosubstrates between the bulk solution and gel phase (Cho & Swaisgood 1974; Levi 1975; Koch-Schmidt 1977). In contrast, the apparent K_m values for coenzyme are invariably 1–100 times greater than the K_m of the free enzyme and are almost certainly attributable to diffusional resistance of the relatively bulky coenzyme molecule (Millis & Wingard 1981).

The data contained in the papers cited in table 1 also dispel the widely held view that immobilized enzymes are more stable to thermal inactivation than their soluble native counterparts. It is clear that in many cases (Manecke & Günzel 1962; Melrose 1971; Kelly *et al.* 1977; Tischer *et al.* 1980; Johnson & Brougham 1981) immobilized oxidoreductases display markedly less stability than their native equivalents, whereas in other cases stabilizations of up to five-fold (Carrea *et al.* 1982) and in one case 25-fold (Lowe 1977*c*) are claimed. However, it should be realized that the reports cited in table 1 represent immobilization by a variety of coupling chemistries to a number of support matrices. In most cases, with very few exceptions (Lowe 1977*a–c*) the detailed nature of the coupling chemistry has not been established, despite the fact that these parameters are known to be of great significance in determining the properties of the resulting immobilized enzymes.

The effect of chemical modification on the activity and stability of oxidoreductases

The nature of the chemical modification of an enzyme before its immobilization can significantly influence its catalytic activity and stability (Kapmeyer & Pfleiderer 1977; Tuengler & Pfleiderer 1977; Klibanov 1979; Torchilin *et al.* 1978; Minotani *et al.* 1979). For example, it is well established that amidination of surface lysyl residues in pig heart lactate dehydrogenase with substituted imidates results in derivatives displaying increased thermal stability without dramatic losses in specific activity (Kapmeyer & Pfleiderer 1977; Tuengler & Pfleiderer 1977; Minotani *et al.* 1979). The increased thermal stability of the amidinated enzyme is believed to be due to the transformation of ε-amino groups (pK_a 10.5) of surface lysyl residues to guanidino groups (pK_a 12.5) because it mirrors the changes observed in the amino acid compositions of mesophilic and thermophilic enzymes (Zuber 1978). The enhanced stability of the modified enzyme may be the result of (*a*) the shift in net charge, and hence conformation, of the protein, (*b*) the formation of new salt bridges because the loci of the individual charges are displaced by almost 0.15 nm on amidination (Kapmeyer & Pfleiderer 1977), (*c*) immobilization or rigidification of the water layer around the protein surface, or (*d*) the formation of cross-links by side reactions (Sekiguchi *et al.* 1979). In marked contrast, acylation of lactate dehydrogenase reduces both the specific activity and the thermal stability presumably because of the loss of positive charge on the lysyl residues and the consequent diminution in salt bridge formation and water layer stabilization. Figure 1 illustrates the thermal stability of pig heart lactate dehydrogenase modified by amidination with methyl 3-chloropropionimidate and by acylation with acryloyl chloride. The stability of lactate dehydrogenase was increased 2- to 5-fold on amidination whereas acylation with acryloyl chloride resulted in an eight-fold destabilization. The large difference in thermal stabilities of the lactate dehydrogenase derivatives underlines the import-

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ance of selecting a modification reagent that will enhance enzyme stability without impairing the catalytic activity. In lactate dehydrogenase and apparently other oxidoreductases such as yeast alcohol dehydrogenase and pig heart malate dehydrogenase, it appears that reagents that maintain the surface charge are preferable to reagents that neutralize the charge. However, some enzymes such as glucose oxidase are destabilized by amidination but show some stabilization on acylation with acryloyl chloride. Similar variability has been observed in citraconylated enzymes, where treatment with citraconic anhydride stabilized α-chymotrypsin but destabilized subtilisin, glucose oxidase and glucoamylase (Barker *et al.* 1979).

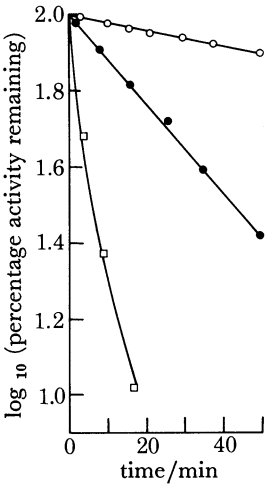

FIGURE 1. The effect of surface chemical modification on the thermal stability of pig heart lactate dehydrogenase at 60 °C and pH 9.0. ●, Native enzyme; ○, enzyme amidinated with 3-chloropropionimidate; □, enzyme acylated with acryloyl chloride.

These results suggest that large oligomeric enzymes such as lactate dehydrogenase, alcohol dehydrogenase and glutamine synthetase (Sekiguchi *et al.* 1979) are more likely to be stabilized with retention of catalytic activity by amidination than by acylation. The increase in net positive charge occasioned by the increased pK_a values of modified lysyl groups may result in greater inter-subunit cross-linking through the formation of salt bridges, thus impeding dissociation into inactive subunits (Chan & Mosbach 1976), or it may accentuate intersubunit interactions through the stabilization of the hydrate layer (Tuengler & Pfleiderer 1977). Not surprisingly, therefore, the extent of surface chemical modification affects the catalytic activity and degree of stabilization of oligomeric enzymes (Lowe 1977*c*; Torchilin *et al.* 1979; Gerasimas *et al.* 1980).

THE EFFECT OF THE NATURE OF THE INSOLUBLE SUPPORT ON THE PROPERTIES OF IMMOBILIZED OXIDOREDUCTASES

In addition to chemical modification *per se*, the physical and chemical nature of the solid support matrix has a marked effect on the activity and stability of immobilized oxidoreductases. The effect of the matrix on the resulting properties of the immobilized preparations has been assessed by interposing a series of spacer molecules between the enzyme and matrix backbone (Taylor & Swaisgood 1972; Lowe 1977*b*). With both lactate dehydrogenase and lipoamide dehydrogenase (Lowe 1977*b*) large changes in the specific activity, thermal stability, kinetic parameters, susceptibility to proteolysis and interaction with monospecific antibodies were

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observed as the spacer length was increased. In both cases the properties of the immobilized enzyme approached those of the native or chemically modified free enzyme as the spacer molecule length was increased, with very little further effects observed with spacers of nominal length 2.5 nm or more. However, lactate dehydrogenase is destabilized when covalently immobilized in close proximity to the matrix backbone (figure 2), whereas the stability of

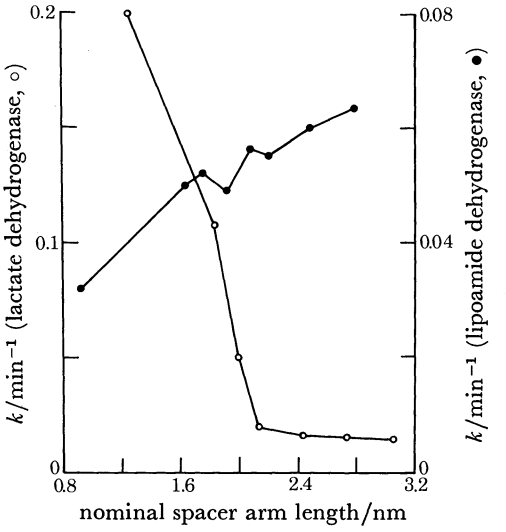

FIGURE 2. The effect of spacer arm length on the thermal stability (thermal inactivation rate constant) of agarose-immobilized oxidoreductases. ●, Lipoamide dehydrogenase at 90 °C and pH 6.50; ○, lacate dehydrogenase at 50 °C and pH 9.0.

lipoamide dehydrogenase is enhanced under similar conditions (Lowe 1977*b*). The reduced stability of immobilized lactate dehydrogenase is probably due to distortion of the three-dimensional structure of the tetrameric protein, because it is accompanied by enhanced susceptibility to proteolysis, altered apparent K_m values and a changed interaction with monospecific antisera. In contrast, it appears that the three-dimensional structure of lipoamide dehydrogenase (Lowe 1977*b*) and some other enzymes (Martinek *et al.* 1977) is rigidified when they are immobilized in close proximity to the matrix backbone, as evidenced by a reduced susceptibility to proteolysis and an increased ratio of diaphorase to lipoamide dehydrogenase activities (Lowe 1977*b*). Nevertheless, lactate dehydrogenase is stabilized some sixfold when attached to the matrix through a spacer arm of at least 2.5 nm length, presumably because deformation of the three-dimensional structure of the enzyme is minimized with a spacer of this length and stabilization is effected by multiple non-covalent interactions with the matrix backbone (Klibanov 1979).

Lactate dehydrogenase displays marked lability when attached to soluble polymers such as agarose and dextran (Foster & Thomson 1973), presumably because rigidification of the tertiary structure of the enzyme is not possible with such polymers. Similar results are obtained on copolymerization of polyvinylated lactate dehydrogenase into polyacrylic gels at gel concentrations above 200 g l^{-1}. Copolymerization of polyvinylated lactate dehydrogenase into polyacrylic gels does not significantly increase the thermal stability of the enzyme unless either a large proportion of the surface lysyl residues have been modified or gel concentrations in excess of 200 g l^{-1} are used. Martinek *et al.* (1977) also found that α-chymotrypsin only acquired appreciable thermal stability when immobilized in polymethacrylate gels at concentrations

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above 250 g l^{-1}. Attachment of lactate dehydrogenase to polyacrylic gels of low conformational rigidity results in destabilization by disruption of subunit interactions. Mechanical rigidity can, however, be conferred by multipoint covalent attachment of the enzyme to more highly cross-linked rigid gels (Lowe 1977*c*; Martinek *et al.* 1977; Koch-Schmidt & Mosbach 1977*a*, *b*). Unfortunately, the specific activity of the enzyme is reduced as the gel concentration is increased, presumably owing to increased diffusional resistance, slowing the rate of diffusion of the relatively high molecular mass coenzyme molecule (Millis & Wingard 1981).

Modification of the matrix microenvironment by incorporating charged, hydrophilic or hydrophobic acrylic monomers into the enzyme copolymers has little effect on the residual specific activity of the immobilized preparations, typically 14–16 %, but can influence the thermal stability of the resulting polymers. For example, a maximum stabilization of 2- to 3 fold was observed with lactate dehydrogenase bound to an acrylamide–acrylonitrile copolymer when a total monomer concentration of acrylonitrile of 50–100 g l^{-1} was present in the copolymer. It is possible that the effect of pendant nitrile groups on the copolymer is analogous to the stabilizing effects observed with aprotic solvents such as acetonitrile and dimethyl sulphoxide (George *et al.* 1969). The immobilization of lactate dehydrogenase to charged polymers, such as those formed by copolymerizing acrylic acid and allylamine into the gel matrix, stabilizes the enzyme in water-miscible organic solvents, urea and salt solutions. In particular, lactate dehydrogenase is highly stabilized in the presence of citrate when copolymerized in a polyacrylamide–allylamine copolymer. In this case, salt-bridge formation appear to anchor the enzyme to the support by multiple non-covalent linkages and thus endows a stable tertiary and quaternary structure on the immobilized enzyme. Similar results have been observed for other Hofmeister anions on the activity and stability of NAD^+-dependent oxidoreductases (Carrea *et al.* 1982).

Coenzyme diffusional limitations

Covalent attachment of oxidoreductases to tightly cross-linked polymers such as polyacrylamide gels not only stabilizes the three-dimensional structure of the enzymes by rigidification of the polypeptide chains but also reduces the specific activity of the enzyme preparations by imparting diffusional resistance to enzyme substrates. A wide variety of kinetic studies with immobilized enzymes over the past two decades has established the fact that the overall kinetics may be dramatically affected by diffusional resistances, either external or internal, to the enzyme support matrix (Engasser *et al.* 1977; Engasser & Hisland 1979). For many of the two-substrate oxidoreductases the coenzyme is freely dissociable and cofactor transport could become the rate-limiting step in the overall kinetics under conditions of slow diffusional transport. With diffusion coefficients of 7.9×10^{-6} cm^2 s^{-1} for NAD^+ (Millis & Wingard 1981) and 3.7×10^{-6} cm^2 s^{-1} for NADH (Kovalenko *et al.* 1981) in immobilized enzyme preparations, it is not surprising that cofactor diffusion could have a marked influence on the initial reaction velocity of enzymatic reactions and thereby reduce the apparent specific activities of immobilized oxidoreductases. Thus diffusional effects could account for the increased apparent K_m values observed for the coenzyme in immobilized enzyme preparations (Cho & Swaisgood 1974; Havekes *et al.* 1974; Smith & Lenhoff 1974; Kovalenko *et al.* 1981; Carrea *et al.* 1982), contribute to the apparent stabilities of the preparations (Engasser & Coulet 1977) and account for the higher specific activities observed with soluble preparations of these enzymes (Foster &

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Thomson 1973) and an almost eightfold increase in specific activity on sonication of lactate dehydrogenase immobilized in polyacrylic gels.

Immobilized coenzymes

The development of efficient enzyme-catalysed processes that require the participation of freely dissociable coenzymes necessitates the development of techniques not only for the immobilization of the enzyme but also for the retention of the coenzyme in the immediate vicinity of the coenzyme-requiring system to maximize the use of expensive coenzyme and for the regeneration of the coenzyme after oxidoreduction. The efficient use of coenzymes in an industrial process can be accomplished in an enzyme reactor designed such that neither enzymes nor coenzymes can escape, while allowing the unimpeded entry and exit of other small coreactants. Simple yet apparently effective techniques for the physical entrapment of unmodified coenzymes include retention in liquid surfactant membranes (May & Landgraff 1976), microencapsulation in semipermeable membranes (Campbell & Chang 1976), and entrapment in hollow fibres (Marconi 1978; Wang & King 1979; Kula *et al.* 1980), ultrafiltration cells (Davies & Mosbach 1974) ion-selective membranes (Rony 1974) or enzyme membranes (Legoy *et al.* 1978, 1980; Wichmann *et al.* 1981). However, conventional entrapment techniques necessitate the use of 'tight' membranes to retain coenzymes and this can lead to severe diffusional restrictions on cosubstrate movement. A widely adopted approach designed to circumvent these difficulties has been to attach chemically derivatized coenzymes covalently to macromolecular 'carriers' (Mosbach *et al.* 1976; Lowe 1979*a*, *b*, 1981). Historically, NAD^+ was first coupled to cellulose (Lowe & Dean 1971), agarose (Larsson & Mosbach 1971) and porous glass (Weibel *et al.* 1971), although significantly higher coenzymic activities could be achieved by covalent attachment to soluble dextran (Lindberg *et al.* 1973; Weibel *et al.* 1974; Lowe & Mosbach 1974; Mosbach *et al.* 1976; Wang & King 1979), polyethyleneimine (Wykes *et al.* 1972, 1975; Zappelli *et al.* 1977, 1978), polylysine (Zappelli *et al.* 1978), polyethylene glycol (Buckman *et al.* 1978; Wichmann *et al.* 1981), soluble polyacrylamide (Muramatsu *et al.* 1977; Furukawa *et al.* 1980) and other polyacrylic matrices (Fuller & Bright 1977).

Immobilization of adenine nucleotide coenzymes may be achieved without impairing the coenzymic activity unduly by inserting a spacer molecule bearing a functionality at positions N^6 or C^8 of the adenine moiety (Mosbach *et al.* 1976; Lowe 1979, 1981). A universal procedure commonly adopted for the synthesis of N^6-substituted derivatives of NAD^+, $NADP^+$, ATP, FAD and coenzyme A involves quaternization of the N^1 position of the adenine ring by alkaline Dimroth rearrangement to afford the more stable and more active N^6-substituted nucleotides (Lowe 1981). Such a procedure may be exploited by traditional in-solution organic chemistry and accompanied by purification of the analogue before attachment to a suitable matrix material (Lowe & Mosbach 1974; Mosbach *et al.* 1976), or it may be effected by solid-phase chemistry (Fuller & Bright 1977). A relatively high molecular mass non-dialysable macromolecular coenzyme derivative such as that illustrated in figure 3 is thus produced.

Properties of polymer-bound coenzymes

In most cases, the physical properties of the matrix-bound coenzymes are not dramatically different from those of the native coenzymes, although there is some evidence from n.m.r.

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spectroscopy that there is a conformational change in the modified coenzyme that could account for reduced activity (Lee & Kaplan 1975). Unfortunately, however, polymer-bound coenzymes suffer varying degrees of lability as a result of the inherent lability of the coenzyme itself and the solvolytic susceptibilities of the linkages between matrix and spacer–coenzyme conjugate and between the spacer molecule and the adenine moiety of the coenzyme. Unfortunately, little quantitative information is available on the stability of immobilized coenzymes, although polyethyleneimine bound $NADP^+$ is claimed to be stable for at least 8 months at 4 °C (Zappelli *et al.* 1977) and polyacrylyl-AMP for over a year at pH 10.0 and 25 °C (Fuller & Bright 1977).

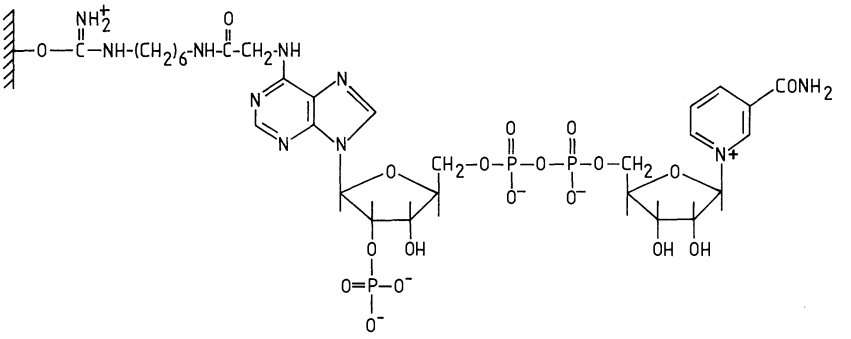

FIGURE 3. The structure of a typical immobilized coenzyme, N^6-[*N*-(6-aminohexyl)carbamoylmethyl]-$NADP^+$ attached to CNBr-activated dextran (Lowe & Mosbach 1974).

The relative coenzymic activities of adenine coenzyme analogues are somewhat lower than those of the parent coenzyme under comparable conditions and decrease further as the analogue is bound to soluble and insoluble support matrices respectively. A number of factors are responsible for this reduction in coenzymic activity. Firstly, the position of derivatization on the adenine base is crucial in determining the coenzymic activity of the resulting analogue, with modification at the exocyclic N^6 amino being recommended on the basis of the limited information currently available and its ease of functionalization (Lowe 1981). However, there is some evidence that with bacterial enzymes C^8 may offer a preferred point of anchorage of the coenzyme to the matrix (Lowe 1979*a*, *b*). Secondly, the nature of the substituent on the adenine ring may either perturb the local architecture of the coenzyme-binding domain or alter the electron density on the adenine ring and thus lead to modified interactions with the complementary coenzyme. Unfortunately, sufficient experimental data are not yet available to permit a detailed assessment of the complex factors influencing the relative coenzymic activity of the derivatives (Lowe 1981). Furthermore, the nature of the matrix to which the coenzyme analogue is attached can introduce steric hindrance and non-specific interactions between the enzyme and matrix backbone and thus lead to reduced relative coenzymic activities. Steric hindrance undoubtedly plays a prominent role in reducing the relative catalytic activity of coenzymes on immobilization. Thus the proportion of enzymically reducible coenzyme depends on the nature of the matrix and linkage; when N^6-(2-aminoethyl)-NAD^+ is attached to an insoluble support such as agarose, only about 5 % of the coenzyme is enzymically reducible even after prolonged incubation with excess alcohol dehydrogenase (Schmidt & Grenner 1976). The proportion of enzymically reducible coenzyme is increased to 40 % on insertion of a long spacer molecule between the coenzyme and support (Schmidt & Grenner 1976) and to more than 90 % when

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the same ligand is attached to a soluble support matrix (Buckman *et al.* 1978). However, the significantly greater retention of coenzymic activity when coenzyme analogues are coupled to water-soluble polymers is not only due to the greater steric accessibility of the ligands but is also to an increased gross diffusional mobility compared with the case where coenzymes are coupled to particulate supports such as agarose. Nevertheless, macromolecular coenzyme conjugates such as NAD^+-dextran still display diffusional impedance and thus reduce the catalytic efficiency of complementary enzymes, particularly those with high turnover numbers (Voss *et al.* 1978). In addition, n.m.r. studies suggest a lower mobility in the transverse plane of the coenzyme moiety in preparations of NAD^+-dextran in such a way as to influence the proper alignment of the coenzyme in its binding domain (Voss *et al.* 1978) and thus reduce coenzymic activity. Finally, multiple non-specific interactions between the enzyme and the spacer molecule or the matrix backbone, or both, can augment those involved in the enzyme–coenzyme interaction and thus alter the rate constants for the immobilized coenzyme from the binary complex (Lowe 1981).

Regeneration of immobilized coenzymes

Since the freely dissociable coenzymes participate as cosubstrates in oxidoreduction, phosphate group transfer or acyl transfer, it follows that the immobilized coenzyme analogue must be repeatedly returned to its original state before further reaction, i.e. recycled to permit the conversion of more than a molar proportion of the substrate into the desired product in the enzyme reactor. As illustrated in figure 4, when the active cosubstrate form of the coenzyme ($coenz_1$)

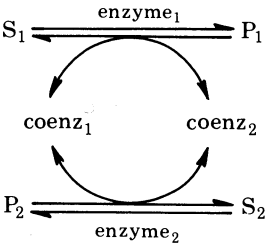

Figure 4. The principle of coenzymic cycling.

participates in the enzyme catalysed transformation of the substrate S_1 into the desired product P_1 it is converted into an inactive form ($coenz_2$) in stoichiometric proportion. If the conversion of active into inactive forms of the coenzyme is coupled to another reaction capable of effecting the reverse reaction, i.e. of reconverting $coenz_2$ back to $coenz_1$, then the attractive possibility exists of producing more moles of P_1 than $coenz_1$. This incorporation of a suitable regeneration step into the reactor converts the cosubstrate into a cocatalyst and thus expedites the production of P_1. Table 2 summarizes the main approaches to coenzyme regeneration, which are applicable to the freely dissociable coenzymes NAD^+, $NADP^+$, ATP and coenzyme A.

It is well known that a number of chemical reagents such as phenazine methosulphate (PMS), phenazine ethosulphate (PES), 2,6-dichlorophenolindophenol (DCPIP), methylene blue, thiazolyl blue and flavins can accept electrons from reduced nicotinamide nucleotide coenzymes. A number of these compounds have been exploited in combination with appropriate enzyme systems to effect the recycling of oxidized and reduced forms of NAD^+. For example, figure 5 illustrates a typical redox dye system used to recycle the reduced nicotinamide nucleotide

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NADH generated in the enzyme-catalysed oxidation of ethanol with PMS acting as the intermediate electron carrier between the reduced coenzyme and the blue dye DCPIP (Campbell & Chang 1976). However, the chemical regeneration of coenzymes with such systems poses some special problems. For example, not only has the specificity of such chemical reagents been questioned, but also the addition of soluble reactants for regeneration leads to contamination of the desired product with recycling reagents. Not surprisingly, therefore, the use of autoxidizable electron acceptors such as acriflavin immobilized to insoluble support matrices has been investigated as a means of reoxidizing NADH without concomitant contamination of the desired product with soluble cycling reagents (Månsson *et al.* 1976).

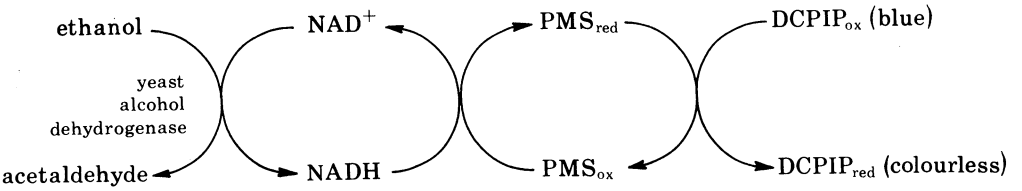

FIGURE 5. Chemical regeneration of immobilized NAD(H) with a redox dye system (Campbell & Chang 1976).

TABLE 2. TECHNIQUES FOR THE REGENERATION OF IMMOBILIZED COENZYMES

chemical regeneration	coupled substrates
electrochemical regeneration	biological methods
coenzymatic cycling	

An alternative approach to the regeneration of immobilized coenzymes involves the electrochemical regeneration of electroactive redox coenzymes such as NAD^+ and FAD (Wingard *et al.* 1982). The electrochemical behaviour of soluble FAD at platinum and graphite electrode surfaces has also been investigated by Braun (1977) and by Gorton & Johannson (1980). It is expected that covalent binding of the isoalloxazine ring of the flavin moiety directly to an electrode surface via a conducting spacer will provide an electron-conducting pathway for electron transfer from the reduced cofactor to the electrode surface. Covalent attachment of flavins via olefin, polydiacetylene, polyvinylpyridine or emeraldine spacers (Wingard *et al.* 1982) or by embedding the enzyme–cofactor in an organic conductor or semiconductor matrix have been suggested and appear to be promising approaches. The electroactivity of the nicotinamide nucleotide coenzymes on most surfaces is rather poor and success with the electrochemical regeneration of enzymically produced NADH has been sporadic. Nevertheless, Aizawa *et al.* (1975) claim a yield of 92% obtained in the oxidation of NADH by an electrolytic method without the use of electron mediators. More recently the electroactivity of NAD^+ has been significantly enhanced by the immobilization of mediators such as 1,2-quinones or naphthalene–stilbene–catechol on the electrode surface (Jaegfeldt *et al.* 1981).

The enzymic regeneration of coenzymes has been under investigation for a number of years and stems from methods developed to amplify very low levels of endogenous coenzymes and thus facilitate their assay. Under optimal conditions with opposing soluble enzymes of appropriate specifi cactivities it is possible to achieve coenzyme cycling rates of 20000–50000 h^{-1} and to detect nicotinamide nucleotide coenzymes down to femtomolar concentrations. More

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recently, however, such methods have been applied to the regeneration of immobilized nicotinamide nucleotide coenzymes (Lowe & Mosbach 1974; Mosbach *et al.* 1976), adenosine phosphates (Mosbach *et al.* 1976), FAD (Zappelli *et al.* 1978) and coenzyme A (Rieke *et al.* 1979), thus maximizing the use made of the supplied coenzyme by transmuting a cosubstrate into a cocatalyst. A

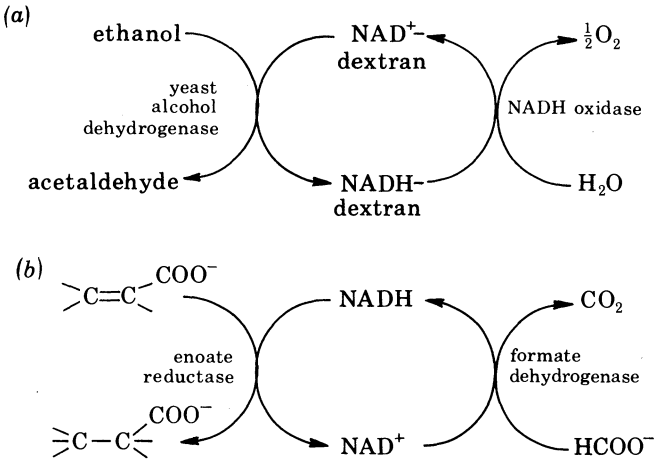

FIGURE 6. Enzymic recycling of immobilized NAD^+ with (*a*) NADH oxidase from *Streptococcus faecalis* (Schmidt & Grenner 1976) and (*b*) formate dehydrogenase from *Candida boidinii* (Tischer *et al.* 1980).

number of systems for the regeneration of nicotinamide nucleotide coenzymes incorporate diaphorase as the return cycling element with ferricyanide, DCPIP, methylene blue or thiazolyl blue as electron acceptor (Lowe 1981). Invariably, however, such systems lead to contamination of the desired product with products derived from the cycling reagents. A number of interesting variants of the enzymatic cycling technique have been proposed which circumvent this problem of contamination. For example, the use of NADH oxidase from *Streptococcus faecalis* (Schmidt & Grenner 1976), formate dehydrogenase from *Candida boidinii* (Kula *et al.* 1980; Tischer *et al.* 1980; Wichmann *et al.* 1981) and NAD^+ peroxidase from *Streptococcus faecalis* (Cox *et al.* 1982) all produce non-interfering products such as water or carbon dioxide in the return reaction (figure 6). An alternative approach to enzymic regeneration of coenzymes exploits the fact that some enzymes display broad specificity and are catalytically active with several substrates such that a coenzyme such as NAD^+ can be cyclically regenerated with a single enzyme (Lowe 1981). For example, horse liver alcohol dehydrogenase oxidizes *trans*-2-decalol, 2-cyclohexanol, 3-methylcyclohexanol, ethanol and propan-1,2-diol to the appropriate ketones with suitable combinations of substrates being used to effect cycling of the coenzyme (Wang & King 1979).

The regeneration of ATP is also a commercially attractive proposition because the syntheses of a number of biological substances involve the consumption of ATP. For example, the total enzymic synthesis of the cyclic decapeptide antibiotic gramicidin S requires the economical regeneration of ATP or ADP (Vandamme 1981). The scheme proposed regenerates ATP from ADP with acetyl phosphate as phosphate donor and *Escherichia coli* acetate kinase as enzyme, with AMP and yeast adenylate kinase as the leading substrate and enzyme respectively. A number of other ATP-regenerating systems have been described in the literature and are summarized in table 3. In many of these, ATP regeneration is effected by naturally occurring

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ATP synthetic systems present in immobilized microbial cells, plant cells or organelles. For example, Samejima *et al.* (1978, 1980) have investigated several systems where this approach is feasible: the production of ATP from AMP and CDP-choline from CMP and choline chloride by *Saccharomyces cerevisiae* cells, the production of coenzyme A from pantothenate, cysteine and ATP and $NADP^+$ from NAD^+ by *Brevibacterium ammoniagenes* and the continuous production of glutathione from L-glutamate, L-cysteine and glycine by *Escherichia coli* cells (Murata *et al.* 1980).

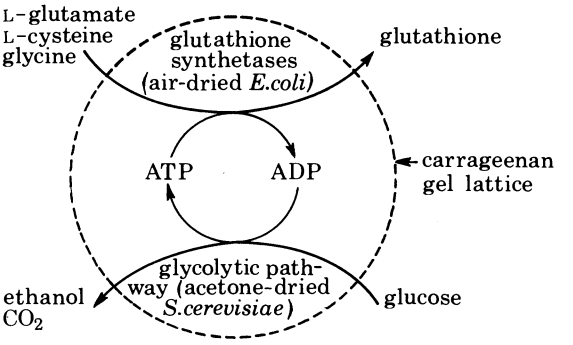

FIGURE 7. ATP regeneration in a reactor for the production of glutathione comprising air-dried *Escherichia coli* cells and acetone-dried *Saccharomyces cerevisiae* cells coentrapped in carrageenan gel (Murata *et al.* 1980).

TABLE 3. REGENERATION PROCEDURES FOR ATP

coenzymic cycling	lettuce thylakoids
yeast cells (glycolysis)	thermophilic blue-green algae
bacterial spheroplasts and chromatophores	mitochondria
chloroplasts	electron transport particles

In the last case, as illustrated in figure 7, a glutathione reactor was produced by coimmobilizing air-dried *Escherichia coli* cells with permeabilized acetone-dried *Saccharomyces cerevisiae* cells in carrageenan gel. The glycolytic pathway was used to generate ATP in yeast cells growing on glucose and subsequently transferred to coentrapped permeabilized cells containing enzyme activities necessary for the desired synthesis of glutathione. A similar approach employing coentrapped cells has been exploited for the biosynthesis of $NADP^+$ and *S*-adenosyl methionine (Murata *et al.* 1980, 1981). Furthermore, the exploitation of cyclic photophosphorylation to generate ATP by the light-driven phosphorylation of ADP has been investigated in immobilized bacterial chromatophores from *Rhodopseudomonas capsulata* (Cocquempot *et al.* 1980), thylakoids (Cocquempot *et al.* 1981) and thermophilic blue-green algae (Sawa *et al.* 1982). In addition, phosphorylating mitochondrial electron transport particles (Matsuoka *et al.* 1981) and immobilized permeabilized plant cells (Felix *et al.* 1981) have been used for the regeneration of ATP and NADP(H) respectively.

COIMMOBILIZED ENZYME–COENZYME SYSTEMS

Coimmobilized enzyme–coenzyme systems require no exogenous coenzyme for activity because the freely dissociable coenzyme is converted into a prosthetic group and thus simultaneously solves the problem of retention and regeneration of the coenzyme. The first indication

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that such an approach was feasible came when coimmobilization of glycogen phosphorylase *b* with an AMPanalogue, N^6-(6-aminohexyl)-AMP, on CNBr-activated agarose, achieved a state of permanent 'allosteric activation' of the enzyme (Mosbach & Gestrelius 1974). Subsequently, lipoamide dehydrogenase was coimmobilized on porous glass with NAD^+ to obviate the requirement for added soluble NAD^+ for activity (Scouten *et al.* 1977), and horse-liver alcohol dehydrogenase was coimmobilized with an NAD^+ analogue, N^6-((6-aminohexyl)-carbamoylmethyl)-NAD(H) on CNBr-activated agarose (Gestrelius *et al.* 1975). In the latter system, the coenzyme was fixed in or near the active site of the enzyme and could be made to undergo redox cycles with suitable combinations of alternative substrates. The 'internal' specific activity in the absence of free NAD^+ approached 40 % of the native free enzyme when the NAD(H) analogue:subunit ratio was 140. An interesting feature of coimmobilized preparations is their enhanced thermal and storage stabilities; after 5 h at 50 °C the internal activity of the alcohol dehydrogenase–NAD(H)–agarose conjugate accounted for 95 % of the total activity compared with only 30 % at time zero.

An alternative but chemically less well defined approach has been to produce enzyme–coenzyme membranes that do not require the addition of soluble NAD^+ for activity (Legoy *et al.* 1978, 1980). In this method, yeast alcohol dehydrogenase or steroid dehydrogenase from *Pseudomonas testosteroni*, NAD^+ and serum albumin were cross-linked with pentanedial to form a membrane of 50 μm thickness. The resulting enzyme–coenzyme membranes were tested on an oxygen electrode; the bound NAD^+ was being regenerated with oxygen, with phenazine methosulphate (PMS) as electron carrier. However, to prevent enzyme inactivation the superoxide anion generated by the reaction of reduced PMS with O_2 must be removed by superoxide dismutase coimmobilized in the steroid dehydrogenase membrane (Legoy *et al.* 1980). Furthermore, attempts to produce coimmobilized systems are invariably dogged by the heterogeneous nature of solid-phase biochemistry, which leads to preparations displaying a variety of properties. Consequently, techniques have been developed to bind suitable NAD^+ analogues covalently to horse-liver alcohol dehydrogenase to convert the freely dissociable coenzyme into a permanently bound prosthetic group (Venn *et al.* 1977; Månsson *et al.* 1978). The analogue N^6-[(6-aminohexyl)-carbamoylmethyl]-NAD^+ has been coupled to the enzyme via a carboxyl group activated with a carbodiimide and *N*-hydroxysuccinimide to yield derivatives containing up to 1.6 mol coenzyme analogue per mole of enzyme subunit. Such derivatives displayed up to 40 % of the specific activity of the native enzyme and exhibited an internal cycling rate of approximately 40 000 h^{-1} in a coupled substrate system. Furthermore, the NAD^+ covalently bonded to horse-liver alcohol dehydrogenase could be cycled, albeit at a slower rate of 300 h^{-1}, with a second enzyme such as lactate dehydrogenase or malate dehydrogenase (Månsson *et al.* 1979). Thus it seems that the main advantages accruing from the enzyme–coenzyme configuration are not only the enhanced stability of the system but also the solution of the dual problem of coenzyme retention and regeneration.

Industrial applications of coenzyme-dependent systems

Although cofactor-requiring enzymic processes are of considerable interest in a number of potential industrial processes, the extension of the scope of practical enzyme reactors from simple hydrolytic reactions has not yet been achieved. So far only exploratory work has been undertaken to assess the feasibility of using coenzymes in model enzyme reactors. Of the systems investigated, the conversion of sorbitol to fructose with sorbitol dehydrogenase (Wang & King

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1979), the conversion of pyruvate to L-alanine with L-alanine dehydrogenase (Davies & Mosbach 1974; Wandrey 1979) and the reductive amination of α-ketoisocaproate to L-leucine with L-leucine dehydrogenase (Wichmann *et al.* 1981), only the last has been assessed over an extended period of time. NAD(H) covalently bound to polyethyleneglycol of molecular mass 10^4 Da, L-leucine dehydrogenase from *Bacillus sphaericus* and formate dehydrogenase from *Candida boidinii* were coretained in an ultrafiltration membrane reactor. L-Leucine was produced continuously by reductive amination of α-ketoisocaproate for 48 days with a maximal conversion rate of 99.9 % and a space–time yield of 324 mmol l^{-1} d^{-1} (42.5 g l^{-1} d^{-1}). An exponential decrease in substrate conversion rate after one month was attributable to inactivation of the coenzyme with a calculated inactivation rate of 16.8 % d^{-1} over the time course of the study. The average turnover number for the immobilized coenzyme was 107 d^{-1}, with both enzymes displaying adequate stability over the same time course. This example therefore represents an attempt to establish the concept of an enzyme–membrane reactor with coenzyme regeneration on a pilot plant scale, and points to coenzyme lability as a major limiting factor. Furthermore, economic analysis of the feasibility of using NAD^+ in enzyme reactors points to cofactor cost as another dominant factor (Lowe 1981).

Biomedical applications of enzyme–coenzyme systems

Additional constraints must be applied when enzymic cycling systems are to be exploited for therapeutic biomedicine *in vivo*. For example, the enzymes must not be antigenic, the consumable substrates (figure 4) are the molecule to be depleted (S_1) and an abundantly available endogenous substrate (S_2) and the products of the coenzymatic cycle, P_1 and P_2, should not be too toxic. In view of these additional constraints, coenzymes have been cyclically regenerated by enzymic systems within semipermeable membranes or hollow fibres. Coenzymes such as ATP, NAD^+ and $NADP^+$ may be recycled within microencapsulated multienzyme systems and be exploited in an attempt to correct enzyme deficiency diseases (Campbell & Chang 1976; Chang & Kuntarian 1978). Thus, in principle at least, the lesion arising from galactokinase-dependent galactosaemia could be corrected with collodion microcapsules coentrapping yeast galactokinase, rabbit muscle pyruvate kinase and pig heart lactate dehydrogenase because galactose 1-phosphate was produced when the microcapsules were immersed in a buffer containing galactose, ATP, phosphoenolpyruvate, Mg^{2+} and K^+. Other coenzyme-containing systems include fibre-entrapped polyenzymatic systems for the depletion of blood ammonia in kidney dysfunction and several enzyme-based detoxification systems designed to mimic hepatic function (Lowe 1981).

Analytical applications

Immobilized coenzyme-dependent enzymes and coenzymes have been exploited in a number of analytical devices. For example, a number of oxidoreductases have been used in flow-through analysers (Gore 1976; Bisse & Vonderschmitt 1978), glucose and lactase dehydrogenases have been exploited to develop substrate electrodes of appropriate specificity (Blaedel & Jenkins 1976; Pfeiffer *et al.* 1980), dextran-bound NAD^+ has been coentrapped with a bienzyme system comprising lactate and glutamate dehydrogenases around an NH_4^+-ion sensitive electrode and used to assay L-glutamate concentrations (Davies & Mosbach 1976), and alcohol and methanol dehydrogenases have been used to construct enzyme fuel cell electrodes (Plotkin *et al.*

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1981; Wingard *et al.* 1982). The enzymic oxidation of methanol to formate with methanol dehydrogenase is especially interesting for the development of fuel cells because the redox reaction is accompanied by the release of four electrons (Plotkin *et al.* 1981).

Conclusions

Despite an increasing wealth of applications in biotechnology, over a decade of research has failed to resolve some of the major practical problems associated with the commercial exploitation of coenzyme-dependent enzymes. Nevertheless, there are encouraging signs that with some enzyme–coenzyme systems economically acceptable pilot plant scale operations are feasible (Murata *et al.* 1980; Wichmann *et al.* 1981) and this should give an added stimulus to future work in this area. For example, a number of areas require special consideration: firstly, the investigation of novel enzymes from new sources that may circumvent the problem of freely dissociable coenzymes by having integral prosthetic groups but able to perform similar catalytic functions. This would include fundamental studies on the immobilization and stabilization of enzymes from thermophilic, halophilic, alkalophilic and acidophilic organisms depending on the process application. Secondly, new methods for the economical synthesis and stabilization of immobilized coenzymes will have to be found in order to circumvent the general weakness of durability under operational conditions (Wichmann *et al.* 1981). Thirdly, new approaches to the synthesis of arrificial redox or transferase activities by recombinant DNA technology or chemical synthesis will have to be investigated. In this context, shifting the equilibrium position of specific phosphatases in favour of phosphorylation by changing the activity of water with immobilized enzymes in water-miscible organic solvents could obviate the requirement for ATP in some circumstances (Antonini *et al.* 1981). Similarly, quasi-biological catalysts such as the immobilized bovine haemoglobin–riboflavin–NADH system for the industrial hydroxylation of pharmaceuticals and for detoxification in extracorporeal shunts (Guillochon *et al.* 1982) and flavopapain as a redox system (Levine *et al.* 1977) should be considered further. Furthermore, entirely synthetic coenzyme model catalysts based on crown ether complexes, micellar systems or transition metal catalysts should be considered as potential alternatives for the catalysis of bulk chemical conversions in the petrochemical or agrochemical industries. By exploitation of these systems it should be possible to mimic the functions of natural biological catalysts and yet improve their durability and economics.

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Discussion

T. K. Sundaram (*Department of Biochemistry, U.M.I.S.T., Manchester, U.K.*). In the early part of his talk Dr Lowe seemed to contrast the multimeric dehydrogenases with their highly hydrated surfaces with the smaller extracellular hydrolases. Did he imply that the hydrolases possess hydrophobic surfaces?

C. R. Lowe. I did indeed contrast the immobilization and stabilization of the multimeric dehydrogenases with the smaller extracellular hydrolases although not, as Dr Sundaram seems to have inferred, in terms of their surface chemistry. The immobilization and stabilization of the low molecular mass single polypeptide chain hydrolases such as trypsin and α-chymotrypsin have been widely reported in the literature and presented as a model for solid-phase enzyme denaturation. However, the dramatic stabilization of such enzymes on immobilization to support matrices appears to be due in part to resistance in unfolding of the enzyme globule by rigidification of the polypeptide chain proximal to the matrix backbone, and in part to reduced autolysis. In addition, modification of surface lysyl groups, for example by acylation with acryloyl chloride, also contributes to reducing autocatalytic destruction of proteases by lowering the number of bonds susceptible to proteolytic cleavage. Furthermore, a comparative study of alkylation and acylation of α-chymotrypsin has shown that a slight increase in thermal stability occurs when up to 80 % of the lysines are modified by either method and that further modification gives a large increase followed by a dramatic decrease in stability. The stability changes were accompanied by spectral changes at 280 nm and indicating an overall change in protein conformation with the appearance of hydrophobic residues on the surface.

The maintenance of charged groups on the surface of the enzyme may be important in some cases, especially if salt links are essential in their stabilization. Lactate dehydrogenase, and a number of the other soluble oxidoreductases, appear to be enzymes for which this is especially important both within and between subunits, because acylation causes a dramatic decrease in thermal stability whereas amidination or guanidation, which maintain or strengthen the surface charge, cause a marked increase in stability. Furthermore, in contrast to the smaller proteases, immobilization of the larger multimeric proteins such as lactate dehydrogenase can allow only a superficial 'freezing' of the polypeptide chains immediately adjacent to the matrix backbone, and some disruption of subunit interactions is inevitable. Thus it is important in attempting to achieve stabilization of oligomeric enzymes such as lactate dehydrogenase that careful consideration be given to the details of the immobilization régime, particularly with regard to the nature of the matrix, the number of linkages and the chemical modification of the enzyme *per se*. These considerations are less critical with the smaller hydrolases.

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