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The Potential in Biotechnology of Immobilized Cells and of Immobilized Multistep Enzyme-Coenzyme Systems [and Discussion]

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The potential in biotechnology of immobilized cells and of immobilized multistep enzyme–coenzyme systems

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Despite the increasing number of reports in the literature on the immobilization of cells or enzymes for the production of more complex compounds involving multistep enzyme systems and coenzymes, no such systems are yet known to be used commercially on a large-scale basis (vaccine production by microcarrier-attached animal cells being an exception). Various systems that have been described are reviewed, including plant and animal cells. Special emphasis is given to the choice to be made between the use of immobilized cells or immobilized enzyme sequences. In this context alternative regeneration methods for coenzymes are discussed. Special attention is brought to the potential for such biosyntheses with immobilized–permeabilized cells. It is likely that the list of immobilized cells and enzymes used at present and restricted to single enzymic steps will be extended in the not too distant future by more complex systems.

1. Introduction

Interest in the use of immobilized cells has grown remarkably during the last decade. This is illustrated by the increasing number of publications in the area. Thus, whereas in 1973 only seven papers appeared on immobilized cells (compared with 220 on immobilized enzymes), 4 years later more than 50 dealt with immobilized cells (Dunnill 1980).

Cells are immobilized by four principal procedures by analogy to enzymes. These are covalent attachment, entrapment (including microencapsulation), adsorption (including bioadsorption to a biospecific ligand such as Concanavalin A) and cross-linking leading to insoluble aggregates (figure 1). Of the various procedures, entrapment within a network of different gels is by far the most widely applied method. A great number of different supports have been used, most of which are listed in table 1, and new suitable support materials are continuously added to this list. Immobilized cells find increasing interest for their potential in the following main areas: (*a*) for the production including transformation of useful compounds, (*b*) in analysis (microbial electrode sensor or microbe thermistor), (*c*) for the removal of harmful compounds such as nitrate from drinking water (see, for example, Nilsson *et al.* 1980), (*d*) as microbial fuel cells (see, for example, Suzuki *et al.* 1980), (*e*) when used in an affinity chromatographic fashion for the isolation of specific compounds such as lectins (see, for example, Ochoa & Kristiansen 1978; Mattiasson & Ramstorp 1980), and (*f*) in medicine. This article concentrates mainly on the first two aspects.

Immobilized cells can be used for the above purposes at different levels of viability (scheme 1). Thus they have found use in the non-viable form obtained for instance through heating or freezing. Permeabilized cells can be obtained under slightly less drastic conditions by brief treatment with organic solvents such as toluene or dimethyl sulphoxide (Felix 1982). In its most sophisticated form, with antibiotics such as nystatin, permeabilization involves the formation

of small pores in the cell membrane, thereby leaving the entire enzyme package intact. The cell membrane of such cells can subsequently be completely reconstituted and the cells made to propagate. Obviously, the borderline between non-viable and permeabilized cells is flexible, but the general advantage gained with these preparations is that inward and outward diffusion of metabolites through the cell membrane, in particular of charged substrates and coenzymes, is greatly facilitated and many of the enzymes tested are left intact. Finally, living cells can be used either under stationary or growing conditions.

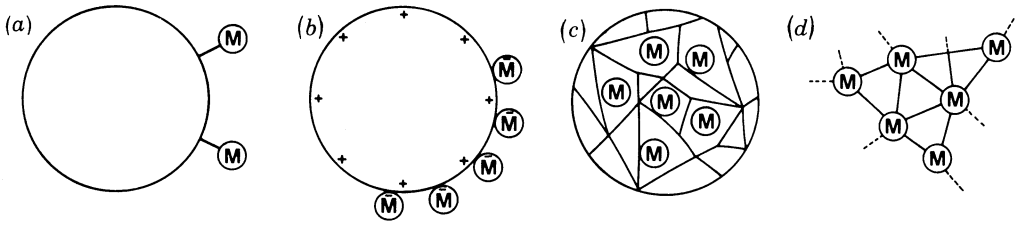
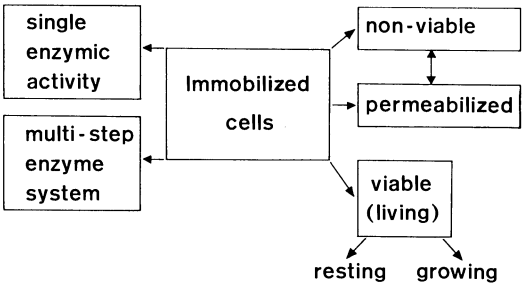


FIGURE 1. Four principal types of immobilized cell preparations used. M, microorganism.

TABLE 1. IMMOBILIZATION PROCEDURES FOR CELLS†

covalent binding		
	hydroxylalkyl methacrylate (glutaraldehyde)	
	carboxymethylcellulose (carbodimide)	
entrapment		
	polyacrylamide	collagen (gelatin)
	alginate	polystyrene
	cellulose triacetate	urethane
	agar	nylon (microencapsulation)
	carrageenan	
	chitosan	
	agarose	
adsorption		
	anion-exchange resin	ion-exchange cellulose
	Dowex 1	polyvinyl chloride and porous bricks
	DEAE-cellulose	
	cross-linked pectate	
	metal oxide	
	bioadsorption: Concanavalin A	
cross-linking		
	glutaraldehyde	
	albumin and glutaraldehyde	
	gelatin and glutaraldehyde	

† Enzymes are immobilized in general by the same procedures and to the same supports, but with major emphasis on covalent attachment to a great variety of supports not listed here (e.g. agarose, porous glass, polyacrylamide derivatives). In contrast, entrapment is less common because of possible leakage.



SCHEME 1

Immobilization of living cells by entrapment for the purpose of producing biochemicals by enzymic conversion was first reported in 1966 by Mosbach & Mosbach, using lichen cells catalysing the two-step enzyme sequence

$$\text{depside ester} \xrightarrow{\text{esterase}} \text{orsellinic acid} \xrightarrow{\text{decarboxylase}} \text{orcinol}.$$

In 1970 it was demonstrated that immobilized cells can be made to grow while present in the support (Mosbach & Larsson 1970). With the system studied, polyacrylamide-entrapped *Arthrobacter simplex* and *Curvularia lunata*, an increased steroid transformation capacity was measured. In the *Arthrobacter* system, catalysing the Δ^{1-2}-dehydrogenation of cortisol to prednisolone, as much as seven times more steroid-transforming capacity was measured after 'reactivation' of the entrapped cells in fresh medium (Larsson *et al.* 1976).

The use of living immobilized cells is now gaining increased importance. They allow the use of multistep enzyme systems (usually involving coenzymes), maximum loading of entrapped cells within the beads can be obtained on growth (in some cases spores of microorganisms have been entrapped because they can stand harsh conditions and as on subsequent growth a homogeneous cell distribution in the beads is obtained (Ohlson *et al.* 1980; Häggström & Molin 1980; Krouwel *et al.* 1981), and they allow 'reactivation' of the immobilized cell preparations after their capacity has decreased by continuous use.

2. PRODUCTION OF BIOCHEMICALS AND ANALYTICAL APPLICATIONS WITH IMMOBILIZED CELLS INVOLVING MULTISTEP ENZYME SYSTEMS AND COENZYMES

A number of excellent reviews have appeared in the literature on immobilized cells during recent years (see, for example, Messing 1981; Bucke & Wiseman 1981; Jack & Zajic 1977; Abbott 1976; Chibata 1979; Brodelius 1978; Linko & Larinkari (eds) 1980; Mattiasson 1979; Birnbaum *et al.* 1982). This brief overview focuses mainly on one specific aspect, namely the use of immobilized cells for the production of compounds, usually involving multistep enzyme systems and coenzymes. Such studies are relatively recent. Despite this, the number of examples found in the literature is already relatively large.

As seen from table 2 they include mainly compounds obtained by synthesis *de novo* starting from simple precursors or nutrients such as glucose, and involve several enzymic steps. Under 'miscellaneous' a variety of compounds is listed, including those involving only one or two enzymic steps but where the participating enzyme system is as complex as in steroid transformations. The reported excretion of α-amylase from immobilized *Bacillus subtilis* is noteworthy (Kokubu *et al.* 1978) because this may be regarded as a model for the future use of immobilized cell systems obtained by recombinant DNA techniques. In this context it deserves mentioning that preliminary results obtained in collaboration with C. Weissmann and H. J. Davies show that both interferon and proinsulin can be formed and excreted by such immobilized cells systems. Although to my knowledge none of the systems listed in table 2 is currently used commercially on a large scale, I am convinced that it is only a matter of time until this will be so; one ought to keep in mind that for each application the superiority of an immobilized system over an already existing conventional and already optimized process has to be proven.

As shown in table 2, immobilized microorganisms have been used most widely. Of more

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recent interest is the use of immobilized plant and animal cells. With regard to plant cells they may find application both for synthesis *de novo* of valuable biochemicals as well as for various transformation reactions including that from digitoxin to digoxin involving 12-β-hydroxylation (Brodelius & Mosbach 1982). Apart from the usual advantages found with immobilized systems, entrapment of the plant cells in, for instance, alginate provides these fragile cells with protection against shear forces. Future developments in reactor design will probably take note of these new properties. Likewise the developments in the application of immobilized animal cells are exciting. Animal cells can be divided into two categories, those requiring a surface to adhere to for growth, so-called anchorage-dependent cells, and those grown suspended freely. Animal-cell cultures have found increasing interest for the production of virus vaccines, interferons, immunochemicals and other cellular biochemicals. Of more recent date is the use of

TABLE 2. COMPOUNDS FORMED BY IMMOBILIZED CELLS INVOLVING MULTISTEP ENZYME SYSTEMS AND COENZYMES

microorganisms	
isoleucine	ethanol, butanol–propan-2-ol
citric acid	glutamic acid
gluthathione	lactic acid (glucose)
penicillin	coenzyme A
cephalosporin	
bacitracin A	
miscellaneous	
hydrocarbon, phenol, benzene degradation	prednisolone (cortisol)
denitrification	acetic acid (ethanol)
plutonium removal	hydrogen gas
cortisol (Reichstein's compound S)	α-amylase
plant cells	synthesis *de novo* of quinones steroids, alkaloids
animal cells	insulin, monoclonal antibodies

microcarriers for anchorage-dependent animal cell cultures. Normally microcarriers based on synthetic polymers carrying charged groups are applied (van Wezel 1967). More recently a natural microcarrier, gelatin in beaded form, which can easily be dissolved by addition of the enzyme dispase for subsequent convenient isolation of cells, has been described (Nilsson & Mosbach 1980). Attempts have also been made to immobilize cells that are not anchorage-dependent by entrapment in various supports (Nilsson & Mosbach 1980; Lim & Sun 1980). Such preparations were shown to produce and excrete monoclonal antibodies (Scheirer *et al.* 1982). The potential of such preparations for direct medical use is well illustrated by the work of Lim & Sun (1980) showing that Langerhans β-islets, microencapsulated in beads of a 'copolymer' of alginate and polylysine, when injected into diabetic rats, were capable of forming insulin, correcting streptozotocin-induced diabetes for 2 weeks.

In the analytical area it appears that the use of microbial sensors for analytical purposes is close to practical application. In one such system described the sensor consists of cells of *Trichosporon brassicae* kept entrapped between a gas-permeable Teflon membrane and an oxygen electrode (Karube & Suzuki 1980). As ethanol formed during fermentation permeates through the membrane, the ethanol will be consumed by the microorganism, leading to a decrease in the dissolved oxygen, giving a steady-state current dependent on the concentration of ethanol. The same combination can be applied to other volatile fermentation products such as acetic

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acid or ammonia. Similarly, with the microbe thermistor device, consisting of, for instance, immobilized yeast cells packed in a small column in the proximity of a highly temperature-sensitive thermistor, changes in medium composition can be recorded (Mattiasson *et al.* 1977). Thus the appearance of a nutrient in the flow-through system or that of a toxic component will lead to an increased or reduced overall metabolic activity resulting in a temperature change in the vicinity of the thermistor.

3. Immobilized multistep enzyme–coenzyme systems

Let us now, taking ethanol formation from glucose as an example, discuss the use of immobilized living cells and alternatives thereto, mainly those involving immobilized multistep enzyme–coenzyme systems. The pathway from glucose to ethanol comprises nine different enzymes as well as the two coenzyme pairs ATP–ADP and NADH–NAD^+. It has been demonstrated by several research groups that immobilized yeast cells form ethanol efficiently from

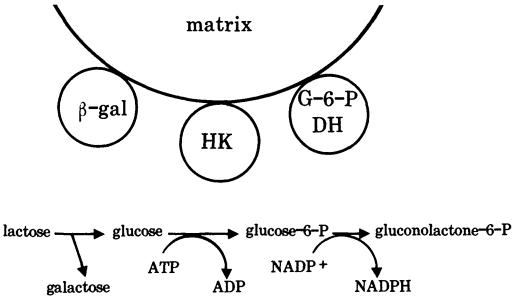

Figure 2. Co-immobilized Sepharose-bound sequential enzyme system (Mosbach & Mattiasson 1978) made up of β-galactosidase (β-gal), hexokinase (HK) and glucose 6-phosphate (G-6-PDH).

glucose. The alternative way would involve first the isolation of all of the different enzymes, followed by their immobilization along with that of the coenzymes. Obviously this appears to be a cumbersome approach. However, in the literature attempts in this more general direction have already been described. Thus a number of sequentially operating multistep enzyme systems have been immobilized, although involving other metabolic sequences, including the system given in figure 2 (Mosbach & Mattiasson 1978). Such systems were shown to be more efficient than the corresponding system made up of soluble enzymes, i.e. the lag phase of the immobilized system before reaching steady state is much shorter. This has been explained as being due to the higher initial concentration of the rate-limiting concentration of the intermediates in the microenvironment of the immobilized enzyme system. With another immobilized system consisting of the sequence malate dehydrogenase – citrate synthase (including lactate dehydrogenase for the recycling of the coenzyme) a constantly higher overall steady-state rate was obtained, probably owing to the fact that citrate synthase bound in the vicinity of malate dehydrogenase shifts the equilibrium towards oxaloacetate formation (Srere *et al.* 1973).

More recently, a four-enzyme sequence (Okamoto *et al.* 1980) and the enzymes of a complete metabolic cycle, the urea cycle, have been co-immobilized to supports (figure 3) (Siegbahn & Mosbach 1982). In the latter case again the immobilized enzyme cycle system was more efficient than the corresponding soluble system, and it was demonstrated that the cycle as such was operative because the amount of fumarate formed after some time was higher than that of

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arginine with which the cycle had been started. Little practical use has yet been made of such immobilized multistep enzyme systems. One such useful application lies in the analytical area where a co-immobilized system composed of glucose oxidase and catalase was shown to give a far higher signal (i.e. ΔT) with the aforementioned enzyme thermistor unit, thus making the analysis more sensitive (Mosbach & Danielsson 1981).

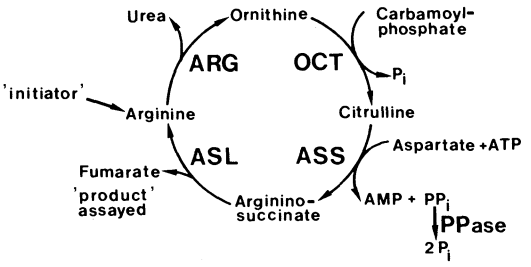

FIGURE 3. Urea cycle: OCT, ornithine carbamoyltransferase; ASS, argininosuccinate synthetase; ASL, arginino-succinate lyase; ARG, arginase; PPase, inorganic pyrophosphatase.

Problems associated with the requirement for coenzymes remain formidable and have been addressed in a number of studies, of which the work on gramicidin S synthesis deserves special mention (Wang *et al.* 1977). The most widely used coenzymes, the adenine nucleotides ATP, ADP, NAD^+, $NADP^+$ and CoA are all extremely expensive and participate in the reactions of one-third of all of the *ca.* 2020 enzymes hitherto allotted a specific number. To allow their regeneration and operation under continuous-flow conditions they too have to be retained, and this can be achieved by their immobilization. To do this, coenzyme analogues have usually to be prepared carrying functional groups such as $-NH_2$ or $-COOH$ because these bind more easily and in a defined way with retained coenzymic activity to supports (Mosbach 1978). Such analogues of, for instance, NAD^+ and ATP are now commercially available (e.g. from Sigma). It is of utmost importance to establish stable linkages between coenzyme and support. A recently described immobilization procedure that seems to fulfil this requirement should therefore be mentioned. By this method various hydroxyl groups carrying supports are activated by using sulphonyl chlorides such as tresyl chloride (Nilsson & Mosbach 1981). On subsequent nucleophilic substitution with, for example, the amino group of an affinity ligand, stable $-CH_2-NH-$ linkages are formed (figure 4). This same procedure, allowing efficient coupling of enzymes at neutral pH, has recently also been applied to enzymes such as restriction enzymes and ligases (Bülow & Mosbach 1982).

Regeneration of the coenzymes can be accomplished by different approaches: chemical, electrochemical or photochemical, and enzymic (Mosbach 1978; Wang & King 1979; Baughn *et al.* 1978; Furukawa *et al.* 1980). Out of these, regeneration with a recycling enzyme or cell is at present the preferred one. Interaction between coenzyme and the enzyme(s) catalysing the reaction of interest as well as with its regenerating enzyme can be allowed to take place in different configurations. When used in bioreactors mounted with an ultrafiltration membrane allowing flow-through operation, a prerequisite is the binding of the coenzyme to a solid or preferentially water-soluble support such as dextran, polyethylene glycol or polyacrylamide to increase its molecular mass for retention. The enzyme(s) taking part, however, do not have to be bound to these supports unless favourable proximity or stabilizing effects are sought (this,

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however, decreases the number of interactions between enzymes and coenzymes). Of the various combinations possible, those best studied involve the use of coenzymes coupled to water-soluble supports and with the enzymes kept in solution. With one such system, alanine formation from pyruvate and NH_4^+ with alanine dehydrogenase was studied with dextran-coupled NAD^+ (Davies & Mosbach 1974) (or polyethyleneimine-coupled NAD^+ (Marconi *et al.* 1975)) and lactate dehydrogenase as recycling enzyme (figure 5). The same dextran-coupled NAD^+ preparation, when entrapped by a dialysis membrane surrounding the tip of an electrode

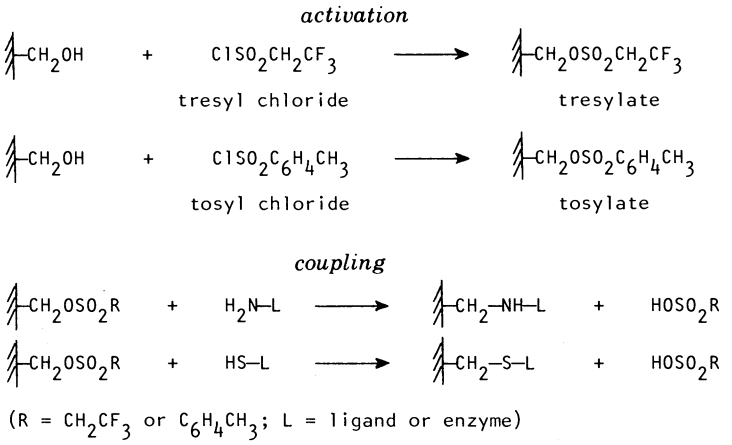

FIGURE 4. Covalent coupling of enzymes or affinity ligands to supports carrying hydroxyl groups by using sulphonyl halides (Nilsson & Mosbach 1981).

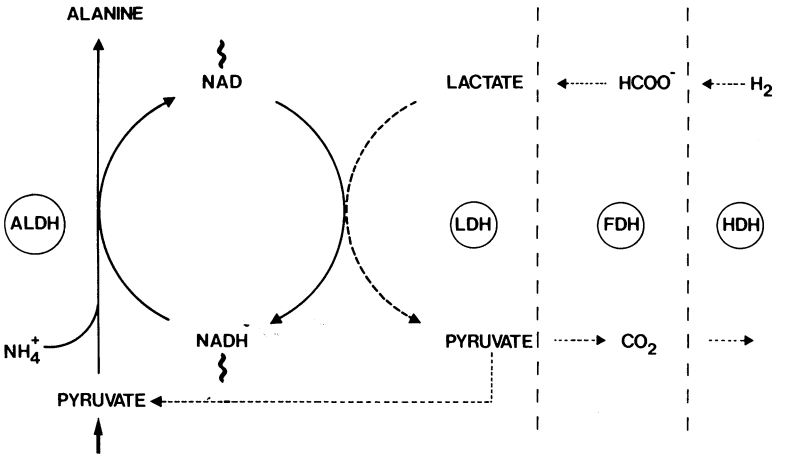

FIGURE 5. Studies with alanine formation from pyruvate, NH_4^+ and NADH by alanine dehydrogenase (ALDH) as the model system. Reactions were carried out in an ultrafiltration chamber with NADH bound to water-soluble supports. Regeneration was carried out with one of the enzymes lactate dehydrogenase (LDH), formate dehydrogenase (FDH) or hydrogen dehydrogenase (HDH).

together with glutamate dehydrogenase and lactate dehydrogenase, could be put to use in analysis. Thus with such an enzyme electrode system glutamate could be determined by the formation of NH_4^+ in an assay mixture containing pyruvate required for the oxidation of the reduced dextran-bound NAD^+ formed (Davies & Mosbach 1974). A similar system used by other workers but with polyethyleneglycol-bound NAD^+ instead and formate dehydrogenase as recycling enzyme, provides the additional advantage that the system is not contaminated with

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the product of the recycling enzymes, CO_2 (Wickmann *et al.* 1981). In this study the enzyme L-leucine dehydrogenase was used to form L-leucine from α-ketoisocaproate producing 42.5 g l^{-1} d^{-1} in the membrane reactor applied. Another such ideal recycling enzyme for NAD^+–NADH is hydrogen dehydrogenase (F. Winqvist, B. Danielsson and K. Mosbach, unpublished work; hydrogen dehydrogenase activity of immobilized cells has also been used for the same purpose (Klibanov & Puglisi 1980)).

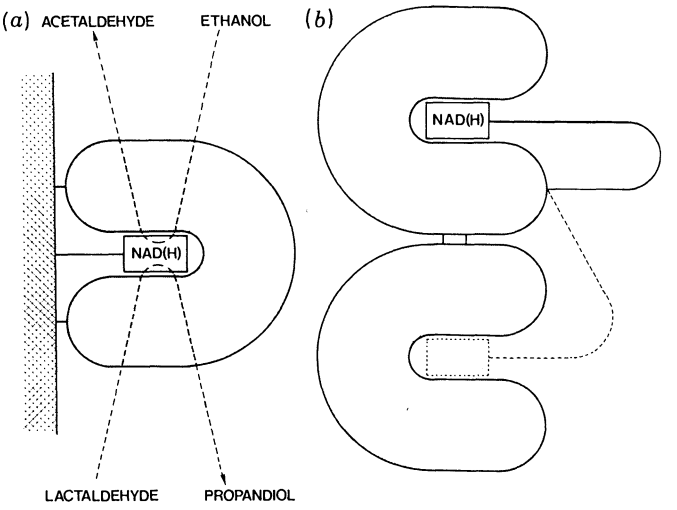

FIGURE 6. Schematic drawings of two ways (*a*, *b*) of 'permanently fixing' the coenzyme NAD(H) in the vicinity of the active site of liver alcohol dehydrogenase. In both cases the NAD^+ analogue used was N^6-[$NH_2(CH_2)_6NH$-$COCH_2$]-NAD^+.

Rather specific alternative approaches to the problem of coenzyme regeneration have recently been described (figure 6). Approach (*a*) has been studied with the system alcohol dehydrogenase–NAD^+, whereby the enzyme and the spacer-extended NAD^+ analogue, N^6-[(6-aminohexyl)carbamoylmethyl]-NAD^+, are both coupled to the support. In this case a so-called coupled substrate assay allows continuous regeneration of the coenzyme while 'fixed' in the active site of the enzyme. A regeneration rate for a single coenzyme molecule of about 40 000 h^{-1} has been obtained (Gestrelius *et al.* 1975). In a similar approach the enzymes alcohol dehydrogenase (Legoy *et al.* 1980) and myoinositol 1-phosphate synthase (Pittner 1981) have been coupled simultaneously with NAD^+ to serum albumin, the resulting preparation no longer requiring externally added coenzyme. In alternative (*b*), the aforementioned NAD^+ analogue is coupled directly to the enzyme alcohol dehydrogenase in such a fashion that the coenzyme can reach the active site of the enzyme and is active. In other words, a normally free coenzyme has artificially been turned into a prosthetic group. Preliminary results indicate that this same coenzyme can then 'swing out' to interact with the second regenerating enzyme, which is bound to the first enzyme (Månsson *et al.* 1978, 1979). Related to this latter approach is the recently successful co-immobilization of the two enzymes alcohol dehydrogenase – lactate dehydrogenase carried out in the presence of bis-NAD^+ leading to a configuration in which the two enzymes are complexed with the active sites in proximity to one another, allowing efficient recycling of the coenzyme NAD^+ (figure 7) (M. O. Månsson, N. Siegbahn & K. Mosbach, in preparation). In system (*a*), in which lactate dehydrogenase (LDH) had been coupled to liver alcohol dehydrogenase (ADH) in the presence of bis-NAD^+ (by using glutar-

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aldehyde), a major portion of the NADH formed by the oxidation of ethanol was recycled efficiently by lactate dehydrogenase placed next to alcohol dehydrogenase. Only a minor portion of NADH was oxidized by the competing lipoamide dehydrogenase (LiDH). In contrast, in system (*b*), where all three enzymes had been bound at random to the matrix, the proportion of NADH oxidized by lactate dehydrogenase was much smaller.

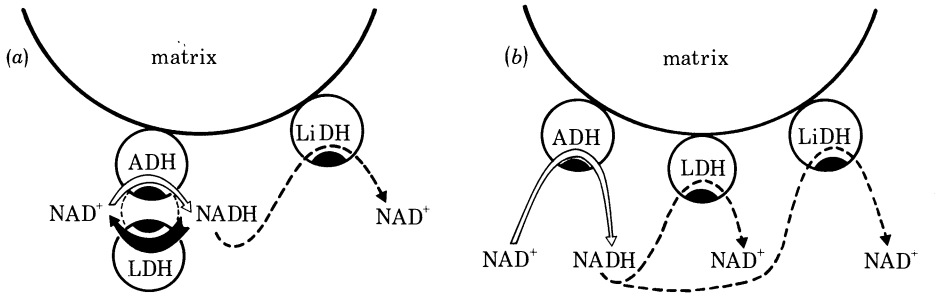

FIGURE 7. The enzymes liver alcohol dehydrogenase (ADH), lactate dehydrogenase (LDH), and lipoamide dehydrogenase (LiDH) were immobilized to tresylchloride or CNBr-activated Sepharose. In system (*a*), only ADH and LiDH were coupled directly to the matrix, whereas LDH was subsequently bound by using glutaraldehyde to ADH in the presence of bis-NAD+. The figure is highly schematic and does not take into account the oligomeric nature of the enzymes involved. The dark areas indicate the active sites of the enzymes.

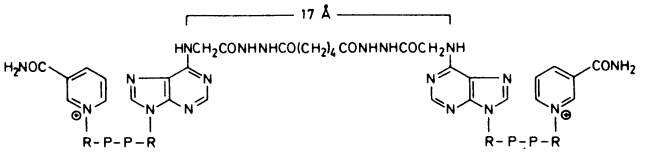

FIGURE 8. The compound bis-NAD+,N_2,N_2'-adipodihydrazido-bis-(N^6-carbonylmethyl-NAD+) used for the 'orientation' of lactate dehydrogenase to alcohol dehydrogenase. (1 Å = 10^{-10} m = 10^{-1} nm.)

In this context it may be mentioned that the bis-NAD+ compound used had originally been prepared to allow enzyme purification by precipitation (now available from Sigma). Thus, addition of this compound (figure 8) to lactate dehydrogenase or glutamate dehydrogenase in the presence of oxalate or glutamate leads to the precipitation of lactate dehydrogenase and glutamate dehydrogenase, respectively (Larsson & Mosbach 1979*b*). Such precipitation occurs within a few minutes (provided that the ratio of subunit to coenzyme monomer is 1:1) and allows the rapid isolation of such enzymes from a crude mixture. Such affinity precipitation greatly resembles immunoprecipitation.

A potentially useful 'intermediate state' between, on the one hand, immobilized cells and, on the other, isolated and immobilized enzymes and coenzymes is represented by cells that have been permeabilized and ideally have also been stabilized by chemical means. One such approach is given in figure 9. With both co-immobilized and permeabilized cells of *Saccharomyces cerevisiae* and *Escherichia coli*, glutathione could be formed on addition of the three amino acids required for the glutathione synthase activity (*E. coli*), whereas the ATP required is generated from the fermentation of glucose to ethanol by the yeast cells (Murata *et al.* 1980). We were recently able to show that this approach can also be successfully applied to plant cells (figure 10). As shown in the figure, the added alkaloid cathenamine is reduced to ajmalicine and NADPH required in the transformation is regenerated by added isocitrate through the isocitrate dehydrogenase found in the same cell (Felix *et al.* 1981). By chemical stabilization methods these systems were recently shown to be active for long periods, up to 2 months. Although such

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immobilized–permeabilized preparations normally have lost their capacity to grow and thus cannot be 'reactivated' in the sense described in the introduction, they may, provided that the enzymes' stability is sufficient, represent an interesting alternative. To allow their use in flow-through systems we have recently immobilized both NAD^+ and ATP to water-soluble polyacrylamide derivatives of molecular mass *ca.* 5–10 kDa and have been able to show that these preparations could enter various permeabilized cells, leading to efficient regeneration (S. Chand & K. Mosbach, unpublished work).

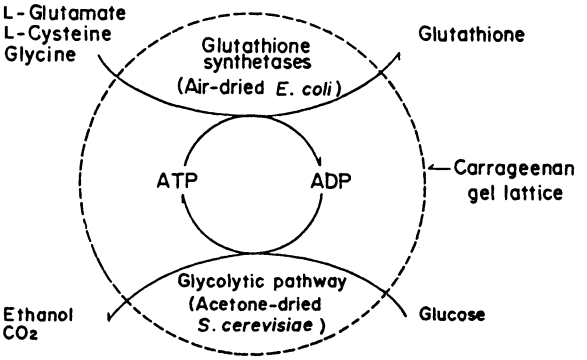

FIGURE 9. Formulation of glutathione by co-immobilized and permeabilized *Escherichia coli* (glutathione synthase) and *Saccharomyces cerevisiae* (regenerating ATP from the pathway glucose to produce ethanol) (Murata *et al.* 1980).

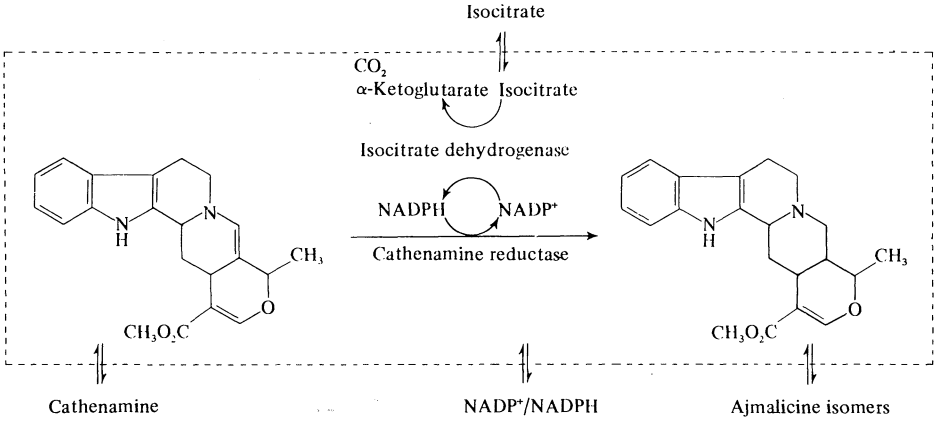

FIGURE 10. Inward and outward diffusion of metabolites in the transformation of the alkaloid cathenamine to ajmalicine in permeabilized and immobilized plant cells of *Catharantus roseus* (Felix *et al.* 1981).

Some additional benefits that can be obtained by some 'tricks', in particular in connection with the entrapment technique, are worth mentioning. Most conveniently this is carried out by simultaneous entrapment of the 'agent' in question together with the cells. For instance, magnetic materials such as Fe_3O_4 particles have been co-entrapped with yeast cells, leading to preparations that are susceptible to a magnetic field; this allows the easy retrieval of the biocatalyst from colloidal and particulate media (Larsson & Mosbach 1979*a*). Likewise MnO_2 or activated carbon particles have been co-entrapped with microbial cells carrying D- or L-amino acid oxidase activity to remove the harmful H_2O_2 formed in the oxidation of essential amino acids to their corresponding α-keto acids, at the same time regenerating oxygen required for the enzymic step (Nilsson *et al.* 1982). For the purpose of generating oxygen required for the same enzymic step, *Chlorella* algae have been co-entrapped with the microbial cells, leading to

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the increased formation of α-keto acids in light (Wikström *et al.* 1982). In another study two different microorganisms, *Gluconobacter melanogenus* and *Pseudomonas syringae*, catalysing the sequence L-sorbose ⟶ L-sorbosone ⟶ 2-keto-L-gulonic acids, were co-entrapped. The *Pseudomonas* cells were added simultaneously to accelerate the rate-limiting oxidation of the intermediate L-sorbosone (Martin & Perlman 1976) (because of the different temperature optima of the two microorganisms, however, the expected rate enhancement was not obtained). Another example is the entrapment of yeast cells within alginate chains carrying the covalently bound enzyme β-glucosidase (Hägerdal & Mosbach 1980). The advantage of such an arrangement is that the glucose formed on the hydrolysis of the substrate cellobiose through β-glucosidase is immediately removed from the system through the yeast cells' converting it into ethanol. Such proximal arrangement reduces product inhibition of the β-glucosidase normally exerted by glucose. A similar co-immobilized cell–enzyme system has been reported, made up of yeast cells and lactase, for the utilization of lactose present in whey (Hägerdal 1980).

4. Conclusion

Known systems based on the use of immobilized cells involving single enzymic steps such as production of L-aspartic acid (aspartase in *Escherichia coli*) or of L-malic acid (fumarase in *Brevibacterium ammoniagenes*) are already in commercial operation. The production with immobilized cells of compounds requiring sequential enzymic steps and coenzymes, however, is not as yet used on a large scale, although it is likely that immobilized living cells and perhaps permeabilized cells will soon find application in this area. Even the simultaneous use of several enzymes together with coenzymes may eventually turn out to become a realistic alternative. This will be particularly true for high-value products, provided that the enzymes and coenzymes participating can be made sufficiently stable. As to enzyme stabilization *per se*, intense research is being carried out (see, for example, Klibanov *et al.* 1978; Martinek *et al.* 1977; Marshall 1979). In our own work we found that enzymes coupled by multiple points of attachment to a support can be made more stable. Thus, ribonuclease A showed increased thermostability by about 10 °C with this approach (Koch-Schmidt & Mosbach 1977), and malate dehydrogenase could be made 'halophilic' by the same methodology, showing enzymic activity in NaCl solutions as concentrated as 4 M (Koch-Schmidt *et al.* 1979).

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Discussion

T. K. Sundaram (*Department of Biochemistry, U.M.I.S.T., Manchester, U.K.*). How easy is it to prepare bis-NAD^+ in the laboratory? Am I right in thinking that the enzyme is precipitated as a ternary complex (abortive) with bis-NAD^+ and substrate analogue?

K. Mosbach. The two adenine nucleotides are linked to one another through a long spacer connecting the N^6-amino groups of both adenine moieties, N_2,N_2'-adipodihydrazido-bis-(N^6-carbonylmethyl-NAD^+). It is not too difficult to prepare bis-NAD in the laboratory (see, for example, Larsson & Mosbach (1979*b*). I should add that Sigma also sells this compound now. The enzyme precipitated as a ternary complex.

P. J. Halling (*Unilever Research Laboratory, Bedford, U.K.*). I would like to ask for Professor Mosbach's comments on an alternative strategy to overcome the problems of cofactor regeneration: to find an enzyme catalysing the desired conversion that does not require complex and expensive co-reactants. Though most redox enzymes known may require NAD^+ or similar complex cofactors, a number will of course react with industrially attractive oxidants or reductants such as O_2 or simple electrode-reactive organic mediators. Similarly, though most synthetic condensation reactions in the living cell are catalysed by enzymes requiring ATP or related compounds, industrially they may be carried out by using hydrolytic enzymes under conditions where the reverse of their normal reaction is favoured (an example was given by Dr Lowe). Even where a suitable enzyme has not been reported for a conversion of interest, a search for a new activity may be a more efficient alternative to work on cofactor recycling with existing enzymes.

K. Mosbach. I understand that what Dr Halling means by a suitable enzyme is one not requiring complex coenzymes like adenine nucleotides. I agree that the line of approach suggested is definitely worth pursuing. Whether it is more efficient I would not know; I feel that both lines of approach, Dr Halling's as well as the one I was discussing, i.e. finding new ways of coenzyme retention and regeneration, should be pursued simultaneously.

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