IMMOBILISED ENZYMES

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I do not intend to burden the reader of this chapter on such a happy occasion as the 100th birthday of Kühne's brainchild with a heavy and complete review of the area of immobilized enzymes. Rather, I feel that I should introduce those readers not too well acquainted with this rather new development with the general methodology involved and illustrate with some examples the various facets of this exciting area. I hope I am excused if I use examples largely selected from our own work; it is made easier for me to get 'the message across' in this way.

It is generally accepted nowadays that few enzymes in vivo actually exist as free protein molecules in an aqueous environment. They are instead either membrane-bound or solid-state assemblies like those found in the mitochondria or are present in gel-like surroundings. One has the impression that this understanding has sunk into oblivion in the hectic research during the last decades directed towards isolation and characterization of a vast number of enzymes. Now it appears, that the cycle is being closed again and we realize more and more that it is time to put the pieces together in order to get an integrated picture for example of a living cell's overall metabolism and its regulation. An expression of this new attitude is reflected both by the increasing amount of work reported on structure and function of biological membranes, but also by the number of recent studies carried out with model systems in which enzymes are immobilized by attachment to or within artificial polymer particles or membranes. These latter studies, to which a large part of this essay will be devoted, have only been made possible through the advances made during the last decade in the technique of anchoring (or immobilising) biological molecules to suitable supports.

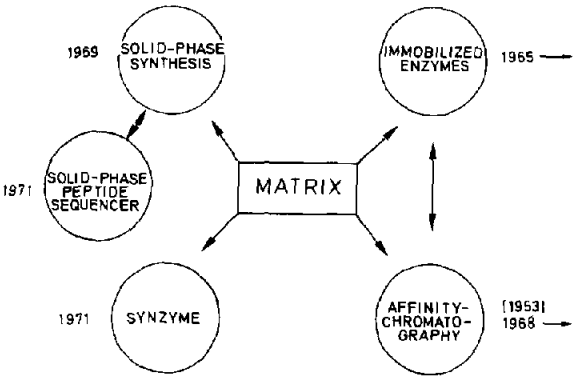

Fig.1. Schematic illustration of the various areas in which solid matrices have found applications. The dates given are approximate and indicate from when various aspects received their greatest attention or when a major break-through occurred.

This technique, which utilizes immobilised biological molecules, has found application in several areas and the facets of this broad field are depicted schematically in fig.1.

(a) The impact of this technique on protein synthesis has been great. A protein with ribonuclease A activity was synthesised in toto by the 'Merrifield' technique employing a solid-phase matrix as a support for the growing polypeptide chain [1]. Proteins possessing the growth-promoting properties of the human pituitary growth hormone (HGH) [2], an acyl carrier protein [3] and proteins with high lysozyme [4] activities have been similarly synthesised.

(b) The reverse of the process depicted in (a), viz. protein sequencing (based on previous work on a 'soluble' procedure [5]) using proteins bound to polymers, has now become automated [6].

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(c) Attempts have been made to mimic the catalytic properties of enzymes by synthesising molecules that incorporate the essential chemical groupings of the active site. Two basic approaches have been followed. The first relies on amino acids to build up a polymer possessing catalytically-important side chains. It involves the preparation of either macromolecular homopolyamino acids [7], which are prepared easily from the reactive *N*-carboxyamino acid anhydrides, or from the less accessible heterocompositional polyamino acids prepared by thermal condensation of amino acids [8]. The second method employs a non-amino acid polymer such as, for example, a polymethylene backbone with the catalytic group attached to it [9]. An active enzyme analog, or 'synzyme' of a proteolytic enzyme has recently been prepared by the latter method. It is capable of hydrolyzing an uncharged nitrophenyl ester in water at a rate $\sim 10^{12}$ faster than that of the uncatalyzed reaction under the same conditions [10]. The authors used a polymethyleneimine framework with methylenimidazole side chains as the catalytic site nucleophiles and containing dodecyl groups to provide apolar binding sites for the substrate. This preparation acted as a 'true' catalyst in that it showed enzyme-like 'turnover' and was effective at pH 7. Phenolic sulfate esters were also very rapidly hydrolyzed with such preparations. In fact, catalytic rates 10^2-fold higher than those observed with the enzyme, II A aryl sulfatase, were reported [11].

(d) Studies on the properties and uses of enzymes bound to matrices have developed rapidly in recent years. Matrix-bound enzymes are usually referred to as *immobilised enzymes.* The advances made during the last ten years represent some of the more dynamic trends of enzymology today and it is to this topic, combined with the related subject of immobilised coenzymes, that this essay will be devoted.

(e) Developments in a closely-related field, viz. the uses of matrices possessing specific groups for purification purposes, have paralleled the advances made in enzyme immobilisation technology. This aspect, known as affinity chromatography, is only touched upon in connection with the treatment on immobilised coenzymes.

Let us first briefly discuss the following questions (1) *what are immobilised enzymes,* (2) *to what supports are they bound,* (3) *how are they bound,* (4) *what are their properties* and finally (5) *to what use can they be put?*

(1) At a meeting on 'Enzyme Engineering' in 1973 under the auspices of the Engineering Foundation Conference, the following recommendations regarding the definition and classification of immobilised enzymes were made [12]. It was agreed to use the term 'immobilised' instead of 'insolubilised', 'matrix-bound', 'solid-supported' and other similar terms. Four major categories of immobilised enzymes can be recognized:

(a) enzymes immobilised by physical or chemical adsorption,

(b) enzymes covalently bonded to soluble or insoluble polymers,

(c) enzymic species arising by crosslinking the protein molecules,

(d) enzymes entrapped into gels or membranes, or within microcapsules.

(2) The spectrum of possibilities encompassed by these four groups is very large. However, at present it is sufficient to state that of the great variety of polymers or matrices which have been employed, the various agarose gels, acrylic polymers and amino-alkylated glass derivatives are the most widely used. In the future, ceramic supports will undoubtedly come into wide-spread use.

(3) It is outside the scope of this essay to describe at great length the large number of immobilisation techniques that are known today. The interested reader is referred to some books and symposia volumes in which detailed information can be found [13–19]. Furthermore, a special volume on immobilised enzymes in the Methods of Enzymology series is in press; this will give experimental details of most of the known immobilisation techniques [20]. In order to illustrate some of the more useful techniques, examples of what are probably the most widely used techniques of covalent attachment of enzymes are given below.

The cyanogen bromide technique [21] involves activation of hydroxyl-group containing polymers such as Sephadex or Sepharose leading to imino-carbonate intermediates to which subsequently the enzyme can be bound (for further reading see in particular J. Porath and R. Axén in [20]). Another widely used procedure involves covalent coupling of enzymes to glass [22]. (For further reading see in particular H. Weetall in [20]). Porous glass is con-

verted to an alkylamino derivative and coupling of the enzyme is then effected with glutaraldehyde. Carbodiimides, preferentially water-soluble derivatives, have also proven of value for attaching enzymes to polymers possessing free carboxyl groups. Physical entrapment of an enzyme [23,24] or of intact microbial cells [24], usually by polymerisation of enzyme–acrylamide mixtures, is another widely employed technique. Prior to polymerisation the enzyme is dissolved in a solution of acrylamide and cross-linking agents such as (bis)-acrylamide. Polymerisation in a two-phase system leads to polyacrylamide–enzyme beads of desired diameter [25].

(4) The effect of immobilisation on enzymes vary of course depending on technique and enzyme chosen. In general it can be said that immobilised enzymes preparations exhibit somewhat decreased apparent V_{max} values and increased K_M values compared to free enzymes usually due to diffusional hindrances exerted by the support. However, as is the case with covalently bound enzymes, the covalent attachment itself may also modify the enzyme. Some 'stabilisation' of immobilised enzymes have been reported.

(5) The final question to be answered is in what areas are immobilised enzymes used. Such preparations are both of *theoretical* and *practical* interest. With regard to *theoretical* aspects, immobilised enzymes represent, as already pointed out in the introduction, valuable model systems, but apart from this the immobilisation technique per se is a tool which can be utilised to solve questions in fundamental biochemistry which cannot be solved, or could be solved only with great difficulty by conventional means. The inherent great potential of this technique is only now being realised and I will therefore devote a rather large part of this essay to some of the few examples so far found in the literature and hope that this will act as a stimulus for future research.

Theoretical aspects

The use of the immobilisation technique per se as tool

As the term immobilisation indicates the freedom of movement of an enzyme molecule can be restricted by this technique. Such a restriction can prevent intermolecular interaction of proteins or subunits but provided sufficient binding is accomplished also stabilise the structure of an enzyme. Let me begin by discussing some of the examples of work carried out in the first category.

(a) Immobilisation allows the study of enzymes under conditions in which they would normally aggregate. Most of the examples given will belong to this category. For instance, when ligand binding is accompanied by association or dissociation of an oligomeric enzyme, immobilisation offers a means for uncoupling this interaction so that those changes associated with ligand binding can be assessed independently from those resulting from protein association. Glutamate dehydrogenase, a hexameric enzyme with a molecular weight of 336 000 and composed of identical polypeptide chains, aggregates in the presence of ADP to linear aggregates with a molecular weight exceeding 2 million. Such polymeric species exhibit higher activity. To determine whether such aggregate formation is a prerequisite for the increased activity observed, or whether ADP acts as allosteric modulator or effector directly on the 'monomeric' species and therefore aggregation only being a secondary phenomenon, bovine liver glutamate dehydrogenase was covalently bound in its 'monomeric' form to porous glass beads [26]. It was found that the immobilised enzyme was also subject to activation by ADP and, since under these conditions no free enzyme was available, the observed activation is independent of association of the active 'monomeric' species.

In another study the effect of aprotic dipolar organic solvents on the kinetics of α-chymotrypsin catalysed hydrolysis was investigated [27]. To prevent aggregation otherwise taking place in a solution containing for example dioxane, the enzyme was bound covalently to porous glass gel. In this study it was then found, that the apparent Michaelis constant K_M and the deacylation rate constant K_3 were markedly dependent on the organic solvent concentration. One other example in which aggregation had to be prevented by the immobilisation technique was in the study of thermally induced unfolding at 65°C of the C-terminus of ribonuclease A using carboxypeptidase. Without immobilisation of the carboxypeptidase used, the latter would have aggregated [28].

(b) Closely related to (a) are examples in which proteolytic enzymes have been immobilised to prevent autodigestion (and also aggregation) otherwise taking

place. For instance, α- and β-trypsin were immobilised to study heat-induced conformational transitions in the temperature range of 20–75°C [29]. It was shown that the unfolding of the enzyme is a multi-state unfolding process in which the biological function is gradually modified and disappears at rather high temperature. The observed breaks in the Arrhenius plots indicated reversible conformational transitions. Again it is almost trivial to state that these conclusions could only be obtained using immobilised enzyme as free trypsin is very rapidly autolysed at elevated temperatures.

Another example of this kind is the study of structural regeneration of chymotrysinogen A in which the protein was immobilised to avoid problems of interactions leading to aggregation and, as a consequence of subsequent trypsin activation, autolysis [30]. Immobilised zymogen was reductively denatured and then aerobically oxidised, giving approximately 80% recovery of esterolytic activity after treatment with trypsin (personal communication). This is in contrast to the 1.4% recovery of trypsin activatable chymotrypsinogen obtained after equivalent treatment in solution. The data obtained with the immobilised system provide us with convincing evidence that the biologically functional conformation of the polypeptide chain comprising chymotrypsinogen A is that of greatest thermodynamic stability. The study discussed here supports similar previous work done on immobilised trypsin [31].

(c) Immobilisation can also be used to prevent the spontaneous association between subunits of an oligomeric protein. In this area really exciting work has been carried out. With this approach it is possible to determine whether the subunit form of an enzyme is catalytically active. If the immobilised subunit is active then comparison of its enzymic properties with those of the corresponding immobilised oligomer can yield valuable information regarding the effects of subunit interactions on enzyme function. Such information is not available for many enzymes. Special techniques have to be used because the native oligomeric structures are sufficiently stable so that severe conditions are necessary to cause dissociation. In these cases, subsequent return to non-dissociating conditions leads to spontaneous reassociation into the native structure. Therefore, the oligomeric and monomeric forms cannot be compared under normal assay conditions. Let me illustrate this point with some examples.

Muscle aldolase was bound covalently to an activated matrix followed by addition of a dissociating agent such as urea whereby the bound subunits remained on the matrix and were prevented from reassociation on subsequent removal of urea. It was found that the individual subunits indeed were catalytically active to some degree [32]. This process is illustrated schematically in fig.2. Results from later studies on aldolase showed that the usually lower specific activity of the bound monomer was not an intrinsic factor but could be increased on using more subtle dissociation agents, indicating that the monomer should have the same specific activity as the tetramer [33]. Similar work was subsequently carried out with transaldolase [34]. Another interesting study in this area is one in which catalytically inactive immobilised monomers of glycogen phosphorylase *b* bound to a matrix were shown to be capable of acting as handle to subunits reconstituting enzymic activities. Further, addition of inactive subunits would even lead to activation of the previously inactive bound subunit [35].

(b) Besides prevention of intermolecular interaction of proteins as in the examples given, the immobilisation technique has also been applied to promote their interaction, for instance in order to obtain stabilisation of protein crystals to be studied with X-ray defraction technique as exemplified in the case of carboxypeptidase A [36]. The advantages gained by such immobilisation using bifunctional agents are the following: the crystals, normally very fragile, become robust and therefore easier to handle. They become completely insoluble under a variety of conditions yet remain permeable to solute. pH and temperature can be varied without dissolution or deterioration.

(e) The examples given under (a) – (d) concern *intermolecular* interactions of enzymes or subunits.

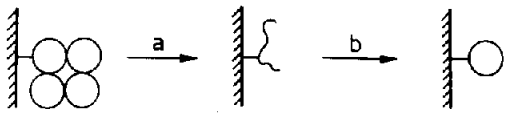


Fig.2. Schematic illustration of the preparation of immobilized subunits of an oligomeric protein. (a) Dissociation utilising a dissociating agent e.g. guanidinium hydrochloride. (b) Removal of dissociating agent leading to refolding of the immobilized subunit.

Some studies on 'conformational' stabilisation of individual enzyme molecules by their attachment to matrices have also been attempted. For instance, the haemoprotein myoglobin has been matrix-bound in its native form as well as in its largely denatured form in the presence of 5% butanol [37]. It was found that both forms, the native and denatured, are to some extent stabilised by linkage to the matrix. Both undergo the reversible transition that occurs in the soluble protein but the equilibria are shifted indicating that the conformations corresponding to the native or denatured forms tend to be 'frozen' by the immobilisation on the matrix. This illustrates the potential of the method to stabilise an enzyme and to study its activity in a specific conformation which in solution is only found under special conditions.

Another way of utilising such stabilisation is in a study on structure–function relationships with immobilised enzymes. In one case trypsin was immobilised within gels [38] or to an elastic support such as nylon. It was found that enzyme activity decreased on stretching the nylon fiber. In the authors' opinion this is due to deformation of the protein molecule (Berezin et al., personal communication). Such mechanochemistry studies would have been difficult to carry out in any other way. The small size of an enzyme molecule makes it impossible to introduce external deforming forces. In another study chymotrypsin was bound to Sephadex and heated above 50°C. Upon cooling another conformation of the enzyme was found, as indicated by a different binding constant of the dye TNS, different maximum fluorescent intensity as well as changes in the catalytic properties [39].

Great caution should be used when interpreting results obtained of this kind, in particular on 'conformational' stabilisation. Nevertheless I am confident that studies of this nature represent only a beginning of what can and will be done in the future. I believe for instance, that we will gain from such studies valuable information with regards to the mechanism of enzyme catalysis.

Enzymes immobilised on matrices as biological model systems

Biological model systems comprised of enzymes immobilised on matrices have been prepared in increasing number in line with the growing understanding that enzymes in the living cell act within the framework of a highly organized structure. Thus it is now understood, as already discussed in the introduction, that most intracellular enzymes function either in an environment resembling a gel, or embedded within interfaces such as membranes or in solid-state assemblages such as those that seem to exist in mitochondria or other organelles of the cell. This point has been convincingly illustrated by the application of centrifugal stratification of the contents of the alga *Euglena gracilis*, which showed that practically all intracellular enzymes were associated with particulate fractions of the cell [40]. In another report is was found that 60% of the enzyme of rat liver cells were bound [41]. As a consequence of a deeper understanding of the in vivo environment of enzymes [19], it is evident that more emphasis is now being put on the study of enzymes under conditions resembling those in the cell.

Immobilised enzyme systems seem well suited for this purpose. Of course it has been argued that immobilising an enzyme, for example, on a synthetic polymer matrix, does not mimic the in vivo situation accurately. However, such studies represent a step in the right direction and will aid us in the necessary 'mental' step to be taken from homogeneous to heterogeneous catalysis. The ultimate goal, should eventually be to reassemble the various parts of the cell, such as proteins, enzymes and membranes, and study them in their natural milieu in order to understand the various facets of a cell's metabolism. However, until we know more about the various components and structures of the cell, use of enzymes immobilised on, or within, artificial matrices as models for cellular components, e.g. membranes, represents an attractive interim phase in biochemistry. One advantage lies in the fact that because of their mechanical stability such preparations are more easily handled than the more fragile natural membranes. Another advantage is that the relatively well-defined chemistry of such matrices permits the study of one parameter at a time such as the effect of hydrophobicity on an enzyme or to ascertain the effect of a charged matrix on enzyme action. Such studies can also provide insights into the kinetic effects of high concentrations in the microenvironment around an enzyme. The influence of two major factors has been studied: one is concerned with the effect of the matrix per se to

which the enzyme is bound and the other with the effects exerted by the products of the enzymic reaction itself.

Microenvironmental matrix-effects

It was shown that the pH-activity profiles of polyanionic derivatives of several proteolytic enzymes, such as the ethylene maleic acid copolymer derivative of trypsin, are displaced by 1 to 2.5 pH units towards the alkaline side as compared to the native enzyme [42]. Polycationic derivatives of the same enzyme exhibited the reverse behavior displacing the pH optimum to more acidic pH values [43]. Also, the apparent Michaelis constant of the positively charged substrate benzoyl L-arginine amide decreased by more than one order of magnitude using the polyanionic derivative of trypsin while the K_M of an uncharged substrate was unchanged [44]. These effects have been interpreted to be due to an unequal distribution of protons or charged substrates between the polyelectrolyte phase or compartment, within which or to which the enzyme is bound, and the outer solution.

Since lipids are abundant in all biological membranes, effects of lipids on enzymic activities are of importance. Such requirements may be due to either specific enzyme–lipid interaction or due to the need for the establishment of a lipophilic or hydrophobic medium as a suitable nonaqueous phase. One study the effect of hydrophobicity on enzyme action was carried out in which alcohol dehydrogenase was immobilised within matrices of varying degree of hydrophobicity (using acrylamide/methylmethacrylate copolymers). With the substrate *n*-butanol, the apparent K_M values shifted to lower values as the enzyme was bound to the more hydrophobic copolymer preparations [45].

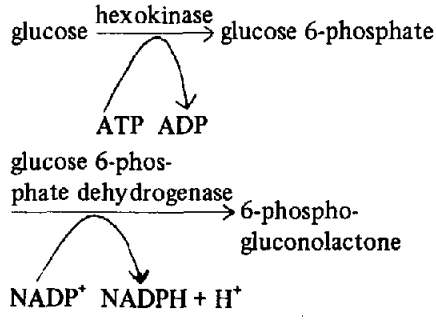
Microenvironmental effects due to enzymic activity

Studies have also been carried out to evaluate the effects on an enzyme of different local concentrations of substrate, product, protons, effectors, etc. produced by the catalytic activity of an enzyme or its neighbouring enzymes, thus creating a different microenvironment for an enzyme. With neutral substrates which give rise to ionized products it was shown, using for example urease cross-linked into an albumin matrix, that the pH in the interior of the membrane increased significantly [46]. When a reaction producing protons in a collodion membrane containing papain was studied, the reverse effect was observed [47]. The changed pH dependence of membrane-bound acetyl choline esterase compared to soluble esterase could thus be explained by such effects, that is, local pH changes in the vicinity of the membrane-bound enzyme as a consequence of hydrolysis of substrate [48].

In the above experiments effects on an enzyme by changing proton concentration produced by the enzyme itself were measured. Since many enzymes produce or utilize protons it is possible that such action could modify other enzyme activities and thus regulate metabolic pathways. A model system containing entrapped hexokinase, glucose oxidase, and trypsin was studied [49]. At an assay pH of 8.6 (the optimum for hexokinase) 15% of added glucose was phosphorylated and the remainder oxidized by glucose oxidase (pH optimum 6.6). A trypsin substrate was then added. Its cleavage produced produced protons in the microenvironment of the two glucose-utilizing enzymes (the external pH was kept constant at 8.6). This acidification of the microenvironment caused a decrease in the hexokinase activity and a simultaneous increase in glucose oxidase activity so that all of the glucose was now oxidized. Nothing definite, however, can be said at this time about in vivo proton concentration as a regulator of enzyme activity since not much is known about buffer capacities present within the cell. Point to point variations make the meaning of the gross-intracellular pH even of a moderate-size cell unclear [50].

Multi-step enzyme systems

The immobilisation of two sequentially working enzymes on the same matrix has been attempted to serve as a simple model for in vivo situations, where enzymes are arranged in consecutive series on membranes or within gel-like structures. The first such two-step enzyme system described was the following [51].



The enzymes were immobilised by covalent binding to CNBr-activated Sepharose or by entrapment within crosslinked polyacrylamide. The total activity of the coupled enzyme sequence was measured, followed by determination of the activities of the separate enzymes. The observed rate of the overall reaction was compared with that obtained using amounts of free enzyme equivalent in activity (units per volume incubation solution) to those of the enzymes of the immobilised system. Another reference system consisted of the individual enzymes immobilised on separate polymer particles.

The results from these experiments are shown in fig.3, where the formation of NADPH is plotted against time. Both the systems in which the enzymes were bound to the same polymer matrix and the equivalent free system showed a lag phase prior to reaching a steady-state level of NADPH production. The immobilised system, however, reached steady-state much faster than the corresponding soluble one, although both systems eventually reached the same steady-state rate. Enzymes immobilised on separate particles behaved identically to the soluble system.

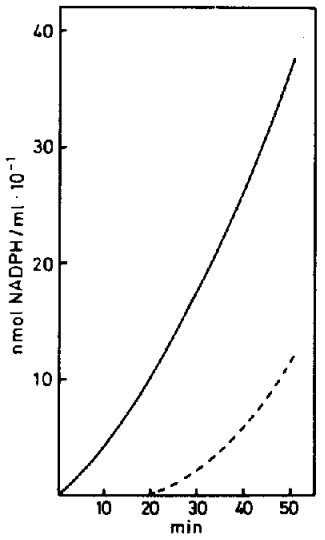


Fig.3. Time course of the coupled reactions of the immobilized (——) two-step enzyme system, hexokinase/glucose 6-phosphate dehydrogenase and its corresponding free (-------) enzyme.

These results were interpreted in the following way. In the immobilised system the product from the first enzyme-catalysed reaction is available in higher concentration around the second enzyme than is the case in the corresponding free system. This is in part due to the close proximity (note that through immobilisation a concentration of different enzymes to each other can be obtained which would be practically impossible to accomplish in free solution) of the enzymes in the immobilised state and in part to the impeded diffusion of the intermediate caused by the Nernst diffusion layer present around the enzyme–polymer particle in stirred solutions [52]. The next enzyme in the sequence thus worked more efficiently and increased the rate of the overall reac-

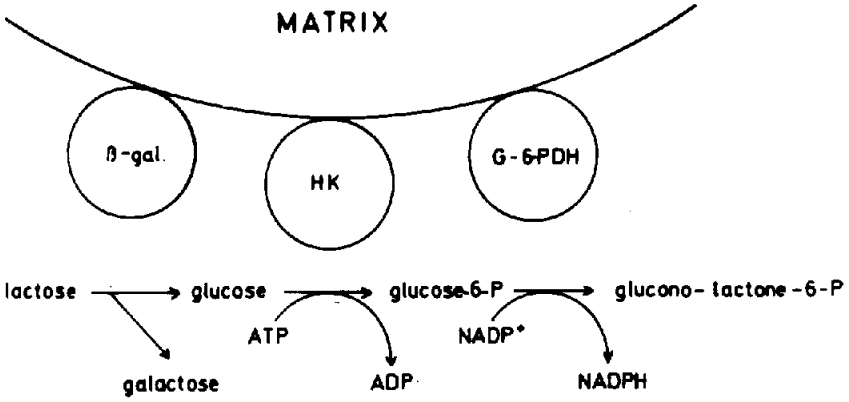


Fig.4. Schematic presentation of the matrix-bound three enzyme system: β-galactosidase (β-gal)/hexokinase (HK)/glucose 6-phosphate dehydrogenase (G-6-PDH) with the reactants.

tion. The behaviour of multi-step enzyme systems, particularly with respect to the length of the lag phase, was greatly influenced by the ratio of the activities of the participating enzymes. Theoretical calculations as well as computer simulations on the behaviour of an immobilised two-step enzyme system were in good agreement with the above data obtained [53]. This two-enzyme system was subsequently extended to a three-enzyme system [54], given in a highly schematic fashion in fig.4.

Again the immobilised system was more efficient in the initial phase than the corresponding soluble system. In fact, the increase in efficiency was even more pronounced than for the two-enzyme system. A general picture of the overall transient rates of consecutive enzymic reactions catalysed by co-immobilised enzymes compared to their soluble counterparts is given in fig.5 [55].

Recently it was calculated, illustrating that such 'model-building' is of biological relevance, that the 'aromatic complex' of *Neurospora crassa* had lags (transient times) that were 10–15 times longer for a hypothetical unaggregated system. From this it was concluded that the aggregated multienzyme system compartmentalises intermediate substrates during the course of the overall reaction [56]. In this context it was pointed out that, in addition to 'channeling' intermediates of competing pathways, reduction of the transient time is an important consequence of the confinement of intermediates within a physically associated enzyme sequence.

One further example of such model studies which has lead to some better insight into the understanding

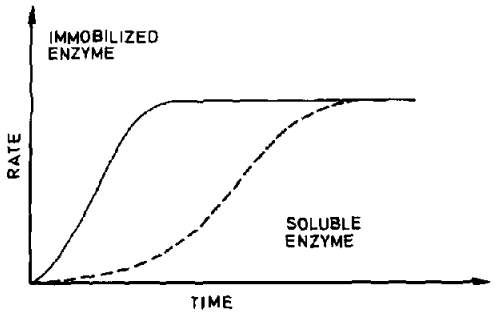


Fig.5. Generalised picture of the system depicted in figs.3 and 5 comparing the overall transient rates of consecutive enzymic reactions catalysed by the co-immobilized enzymes and the enzymes in free solution [55].

of metabolic events is the following: recent reports had supported the older idea that the concentration of oxaloacetate was primarily responsible for the regulation of the rate of oxidation in the Krebs cycle. One of the problems in understanding this regulatory mechanism was that the apparent free concentration of oxaloacetate in the mitochondrial matrix is so low that the rate of its reaction in the citrate synthase reaction would not be commensurate with the known rate of the cycle in mitochondria as estimated from 0_2 utilization. If, however, an organization of Krebs-cycle enzymes existed within the matrix, then it is possible to imagine segregated metabolic pools in which a locally high substrate concentration could be maintained in the region of each enzyme's active site. This could occur in spite of a low (measured or calculated) overall concentration of the substrate distributed over the whole matrix. In order to test the validity of this assumption, the two sequential enzymes of the Krebs cycle, malate dehydrogenase and citrate synthase, were bound in close proximity to each other on a matrix together with a dehydrogenase [57]. It was shown that such a system was more efficient than the corresponding soluble system when measured at low malate concentrations, and strongly suggested that clustering of enzymes belonging to the Krebs cycle has a kinetic advantage. The apparent discrepancies of the previous interpretations may thus be resolved. Subsequent studies with mitochondria which were treated with increasing concentrations of digitonin [58] were in line with the interpretation gained from these model studies. The loss of latency of enzymes observed as the inner membrane became permeable to substrates and acceptors was in line with the picture of compartmentation of the enzymes of the Krebs cycle in the mitochondrial matrix.

Miscellaneous

Other interesting model systems are the so-called 'artificial enzyme membranes'. For instance, in one such system, hexokinase and phosphatase were co-crosslinked with an inert protein to form membranes [59]. The two different enzyme layers were then sandwiched between two selective films impermeable to glucose 6-phosphate. Both layers were impregnated with ATP and placed between glucose solutions. In the first layer glucose is a substrate, and the glucose 6-phosphate formed diffuses along its own concen-

tration gradient into the second layer where glucose is a product. In this matter a system acting as glucose pump was accomplished.

Another example in which the influence of membrane structure was studied, was the two-enzyme system, xanthine oxidase–uricase in which xanthine, the substrate for enzyme number one, acts as competitive inhibitor for the next enzyme [60]. It was shown that the uricase reaction rate at steady state was higher than with the free enzyme in solution.

It is tempting to speculate in what direction this 'model building' will go. It is not unlikely that since it lies within the reach of present day immobilisation technique, that not only parts but complete metabolic cycles will be immobilised on matrices and it may very well be that totally new artificial metabolic cycles are established. In so doing we are leaving the original purpose in designing the model systems discussed, i.e. to obtain, as the name implies, models of biological systems and we enter the field of what may be called 'synthetic biochemistry' to which I will devote some concluding remarks at the end of this chapter. At this point I wish to stress that insight gained from these studies of immobilised multi-step enzyme systems are also of practical value. Thus it was shown recently, that for the scaled-up production of gluconic acid from glucose utilising the two enzyme system glucose oxidase–catalase, arrangement of the enzymes in proximity to each other, on the same particle, was advantageous over that of their immobilisation to separate particles [61]. In this case the advantage gained was the local enrichment of the catalase substrate, oxygen. This then may lead us to a discussion of some of the advances made in the practical application of immobilised enzymes.

Application of immobilised enzymes

Immobilised enzymes have been proven to be of practical value in the following four areas: (1) enzyme technology also referred to as enzyme engineering (2) analysis (3) medicine and (4) 'organic' chemistry.

Let me illustrate with a few examples the dynamic development that has taken place in the above areas during the last few years. First, however, I would like to summarise some of the advantages involved in the use of immobilised enzymes or cells over that of soluble enzymes: (a) they can be reused, (b) the usual separation problems of catalyst from product are practically eliminated, (c) their reactions require less space, (d) better control of the reaction is possible, (e) they are applicable to continuous flow-systems and (f) immobilised enzymes have in some cases been shown to have better 'stability' in its broad sense than soluble proteins. In addition, in the medical applications, adverse immunological reactions can only be prevented by encapsulation or entrapping of the enzymes used.

Enzyme technology

A number of processes employing immobilised

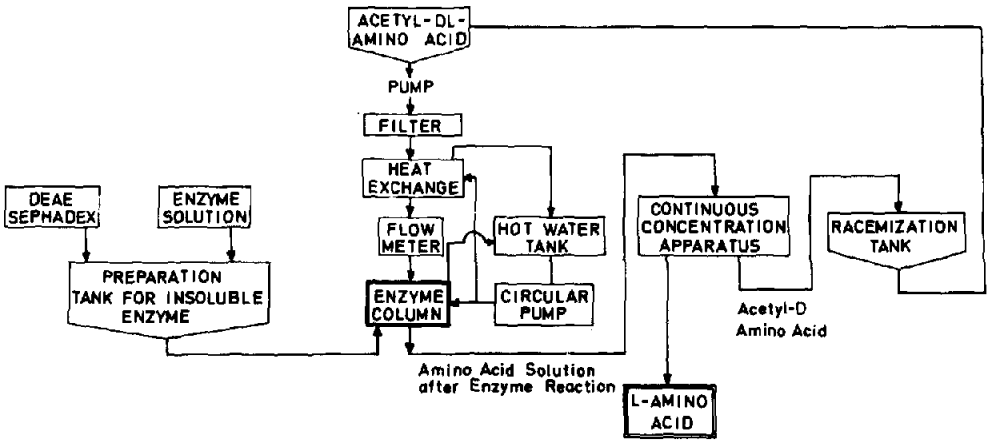


Fig.6. The aminoacylase process.

enzymes or intact micro-organisms on a technical scale have come into use in recent years. These include the resolution of racemic amino acid mixtures into their optical antipodes for the production of, for example, L-DOPA or L-methione using immobilised aminoacylase in a fully automated process in enzyme reactors with a reported capacity of ∿ 700 kg per day [62] (fig.6). The overall operation costs of the immobilised enzyme process were reported to be about 60% of that of the conventional batch process using native enzyme. Furthermore, a process yielding 6-aminopenicillinic acid for the preparation of semi-synthetic penicillins from penicillin using immobilised penicillin amidase is in operation [63,64]. Immobilised whole cells entrapped in polyacrylamide gel networks are used on an industrial scale for the production of L-aspartic acid which involves passing a solution of ammonium fumarate through a column containing entrapped *Escherichia coli* cells [65]. The application of fiber-entrapped β-galactosidase for the reduction of the lactose content of milk to overcome the problem of lactose intolerance has now left the pilot-plant stage [66].

In the food industry great efforts are made to utilise immobilised enzymes. Probably the most outstanding example of the commercial application of an immobilised enzyme to date is the production of high-fructose corn syrups, using immobilised glucose isomerase converting D-glucose to D-fructose. In the process employed by Clinton, Corn Processing, USA, a 93% glucose solution is isomerized to about 42% fructose with a current capacity of 500 million lb per year, the product competing economically with the invert sucrose made from cane and beets. A total of approximately 1 billion lb of high fructose syrup from starch were produced in 1974, using the three-enzyme process α-amylase–glucoamylase–glucose isomerase, making the process the largest volume use of immobilised enzymes in the world (K. Pye, personal communication).

Steroid transformation processes applying immobilised enzymes or micro-organisms have also attracted attention in the light of the wide use of corticosteroids in the treatment of rheumatoid arthritis [67]. However, in this area, in particular on enzyme stabilisation, much work remains to be done. One such example is given in the following scheme:

$$\text{Reichstein S} \xrightarrow[\text{of the steroid}]{\text{11-}\beta\text{-hydroxylation}}$$

$$\text{cortisol} \xrightarrow[\text{of ring A in the steroid}]{\Delta^{1-2}\text{ dehydrogenation}} \text{prednisolone}$$

For the conversion of Reichstein's compound S to cortisol, fungal cells of *Curvularia lunata* were used entrapped in polyacrylamide because of the labile nature of the 11β-hydroxylase. For the subsequent step, either entrapped Δ^{1-2} steroid-dehydrogenase of *Corynebaterium simplex* were used in conjugation with phenazine methosulfate as an artificial electron acceptor or intact cells.

Analytical area

In the analytical area the application of immobilised enzymes has made immense progress. Particularly noteworthy are the developments leading to enzyme-electrodes [68,69] enzyme-thermistors [70] and the use of enzymes covalently bound to nylon in automated analysis [71]. In the first two devices the proximity of the 'sensor' (transducer) to the detecting enzyme is utilised to obtain pronounced signals. Thus in an enzyme electrode suitable for analysis of urea, the enzyme urease is 'immobilised' around the sensor, in this case a cation-sensitive electrode (fig.7). A great number of compounds have been analysed with different enzymes and/or electrodes. Development in this area has matured so far that enzyme electrodes are now commercially available. The same basic principle of proximity of enzyme to sensor is also utilised in a measuring unit called enzyme-thermistor, in which the heat of enzymic reactions is registered (fig.8). It comprises one of the most general sensors known and its specificity is obtained by the surrounding enzyme.

In another interesting recent development, an electrochemical cell is utilised as sensor around which the enzyme is immobilised [72]. For instance, cholinesterase is used for monitoring of air and water for the detection of enzyme inhibitors such as pesticides. As an example, paraoxon, an organophosphate, is detected at a level of 0.1 ppm in air and water. The great sensitivity of these systems is due to the principle applied. Thus the enzyme is continuously fed with substrate. The occurrence of traces of inhibitor will dramatically increase the potential since as in the

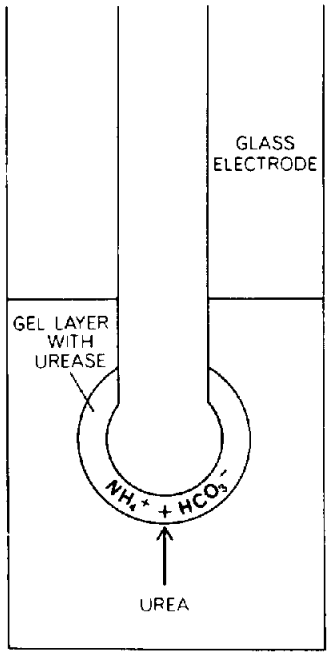


Fig.7. Principle of an enzyme electrode.

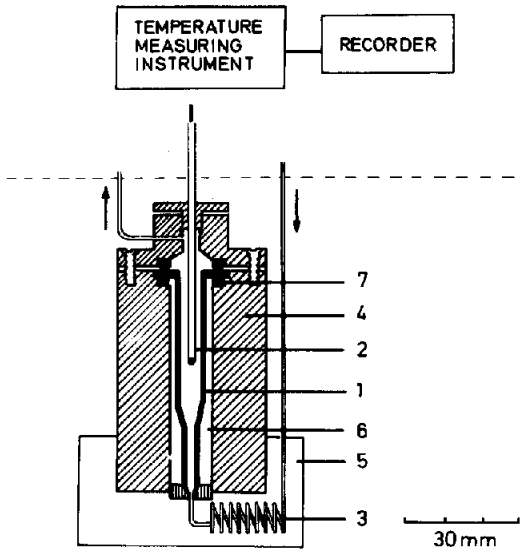


Fig.8. Principle of an enzyme thermistor. 1 = Enzyme column, 2 = thermistor. The entire measuring unit is immersed in a water bath. Substrate solution is passed through a heat exchanger [3].

case of butyrylcholinesterase with a turnover of 84 000, inhibition of the active site reduces the formation of substrate reaction product by 84 000 molecules in tests lasting 1 min. Finally the immunoenzymic technique which makes use of enzymes coupled to antigens or antibodies is becoming increasingly available in biomedical analysis [73].

Medical area

Research on the application of immobilised enzymes in the medical area has been extensive in the last few years. I am thinking in particular on the utilisation of microencapsulated enzymes in therapeutic applications [74] such as the use of urease for lowering blood-urea or the use of enzymes entrapped in liposomes for treatment of lysosomal storage diseases [75]. These enzyme preparations have been administered by different routes such as implantation, by injection, intravenous injection, extracorporeal perfusion or gastrointestinal administration. They have been applied for congenital enzyme deficiency, substrate-dependent tumour, organ failure and metabolic disorders or as red blood cell substitutes.

'Organic' chemistry

Immobilised enzymes are attracting attention also in the field of organic chemistry. In a broad sense the examples given for the use of enzymes in enzyme technology such as the amino acylase process belong to this category. But their potential as tools in research laboratories for both small-scale synthesis of biochemicals and 'true' 'organic' chemicals has only recently been realized.

I am thinking of work done on the study of the biotransformation of drugs, where usually only a few milligrams of a specific metabolite are required, primarily as a reference for the identification of that metabolite in biological fluids or tissues. I am referring to work on an immobilised mixed function hepatitic drug oxidase which has been used in the synthesis of the amine oxides of tertiary amines [76]. Using this method the *N*-oxide metabolites of tertiary amine antihistamine and tertiary phenothiazine drugs have been prepared. Another example of such small scale synthesis is the synthesis of porphobilinogen (from two molecules of δ-aminolevulinic acid) by immobilised δ-aminolevulinic acid dehydratase [77].

Organic chemists are becoming increasingly aware of the potentials of enzymes in particular in the synthesis

of stereospecific compounds. For instance, α-chymotrypsin has been used to resolve over a hundred acids via its catalysis of the hydrolysis of their ester derivatives. Another example is the use of alcohol dehydrogenase in the stereospecific reduction of a keton. Whenever it comes to the ability of discriminating between enantiomers and to distinguish enantiotopic groups and faces of molecules possessing prochiral centers, enzymes will be used. Although immobilised enzymes have so far only been used to a minor extent to carry out these reactions, the inherent advantage of using immobilised preparations should also apply here and will certainly be utilised in the near future [78].

Immobilised coenzymes

A large number of enzymic processes of practical interest require coenzymes. The same reasons that motivate the immobilisation of enzymes apply also to coenzymes and substantial amount of effort has been directed in recent years towards the problem of retention and regeneration of the expensive coenzymes. Besides their utilisation as 'active coenzymes' they find, in particular the various adenine nucleotides, increasing application in affinity chromatography as 'general ligands' [79] and their potential in the study of enzyme mechanism and kinetics has been realized. In common with other ligands attached to matrices, coenzymes often have to be derivatised to introduce functional groups suitable for binding and spacer arms may be necessary to be inserted between coenzyme and matrix to increase their steric availability to the enzyme. Two synthetic approaches have been followed for nucleotide coenzymes:(a) the nucleotide analogue consisting of nucleotide and spacer has been synthesized first and then coupled to the matrix, (b) the nucleotide has been coupled to a preformed matrix which already has the spacer attached. The first mentioned pre-assembly approach is in my opinion advantageous since well defined preparations can thus be obtained, whereas with the other approach unsubstituted spacer arms are likely to be left on the matrix. Of the various adenine nucleotide analogues described in particular NAD(H) and NADP(H), those substituted at the exocyclic amino group [79,81] (fig.9) and those substituted at position C-8 in the adenine moiety [80] retain most of their 'coenzymic activity'. In this essay I will have to restrict myself and I will only give one example to illustrate the usefulness of immobilised coenzymes. The NAD(H)-analogue was used bound to water soluble dextran of molecular weight of approximately 40 000. The preparation was then applied as a component in an enzyme electrode system [82] (fig.10).

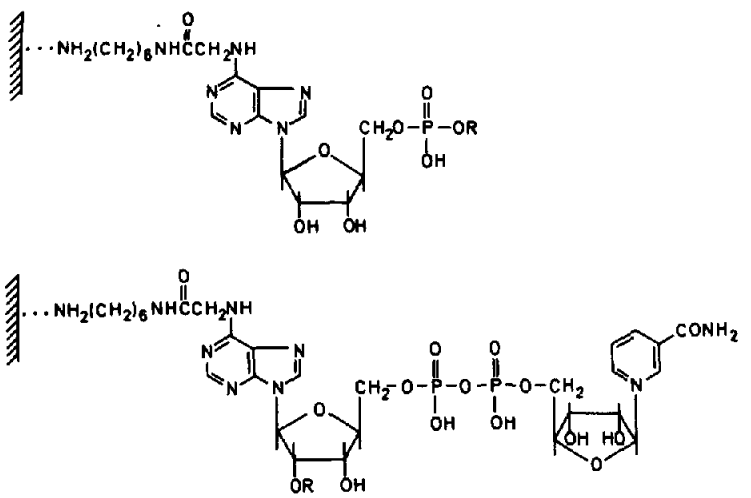


Fig.9. Immobilized analogs of the adenine nucleotide coenzymes. Type A mononucleotides: R=H (AMP), R=HPO_3 (ADP), R=$H_2P_2O_6$ (ATP). Type B dinucleotides: R=H (NAD(H)), R=HPO_3 (NADP(H)). The dotted lines indicate the site of attachment to polymer support, which was achieved by coupling to CNBr activated agarose.

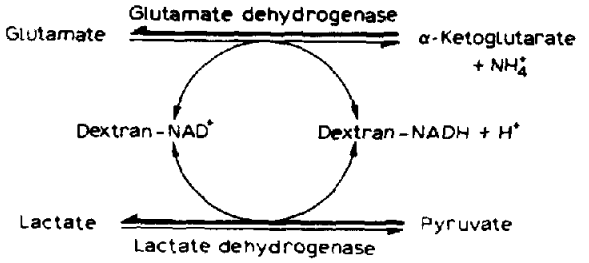


Fig.10. An enzyme electrode system using recycling NAD(H).

In the example given here glutamate concentrations were determined and the electrode consisted of lactate dehydrogenase and glutamate dehydrogenase entrapped as soluble enzymes together with the above dextran-NAD(H) with a dialysis bag placed around a cation-sensitive electrode. The generation of NH_4^+ in the vicinity of the NH_4^+-sensitive electrode caused a millivoltage deflection which was shown to be directly proportional to the logarithm of the glutamate concentration. The same dextran-NAD(H) preparation had also been used as recycling component in enzyme reactor model studies for the synthesis of alanine from pyruvate using the system alanine dehydrogenase–lactate dehydrogenase [82].

Perspectives

I am convinced that development of the various areas discussed, in which immobilised enzymes are put to use, will be even more rapid in the future. Beyond these applications, I feel we will, in analogy to organic chemists who originally were primarily concerned with Nature's own 'Naturprodukte' until they embarked on 'independent' synthetic research, take an 'independent' step in 'synthetic biochemistry'. It is certain that more attempts will be made to obtain enzyme-like catalysts and the various immobilisation techniques applied to enzymes will have impact in this research. But also the kind of 'enzyme-matrix diad' discussed, should be of interest in this context, allowing hundreds of new useful combinations, some of them probably of general applicability and interest and maybe even possessing unexpected properties. For instance, one can envisage changing the apparent properties of enzymes by applying a hydrophobic or charged matrix, unfavourable equilibria of enzyme-catalyzed reactions can be overcome through the binding of several enzymes acting in sequence in close proximity to each other on a matrix. Binding of enzymes in a 'frozen' conformation leading to changed properties can also be envisaged. In addition, one might contemplate coupling enzymes in a metabolic sequence not found in Nature; it should even be possible to create artificial 'cells' accomplishing complete metabolic events and cycles.

I would like to devote the last section of this Perspective to describe modest attempts of this kind of modification or 'synthetic biochemistry' which have been made in these latter directions.

In a study of the enzyme phosphorylase *b*, which has an absolute requirement for the effector AMP, it has been found possible to bind this enzyme to a matrix which had been substituted with AMP in such a manner that no more free AMP was required for activity (fig.11) [83]. Following upon this approach alcohol dehydrogenase was immobilised around bound NAD^+ in such a manner that no more free coenzyme was needed for activity (fig.12) [84]. Regeneration of the reduced coenzyme formed after oxidation of ethanol to acetaldehyde was obtained through the simultaneously added 'alternative' substrate, lactaldehyde. It is likely that for other oxido-reductases similar pairs of substrate can be utilised (or possibly be replaced by artificial electron acceptors and/or donors) to regenerate the coenzyme.

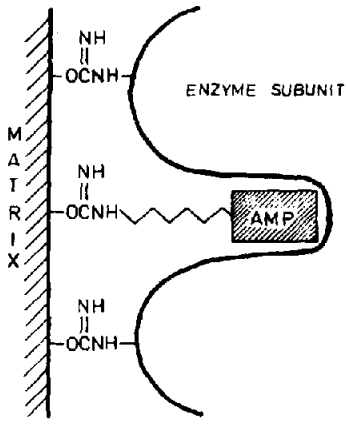


Fig.11. A schematic representation of the active phosphorylase/AMP/matrix complex.

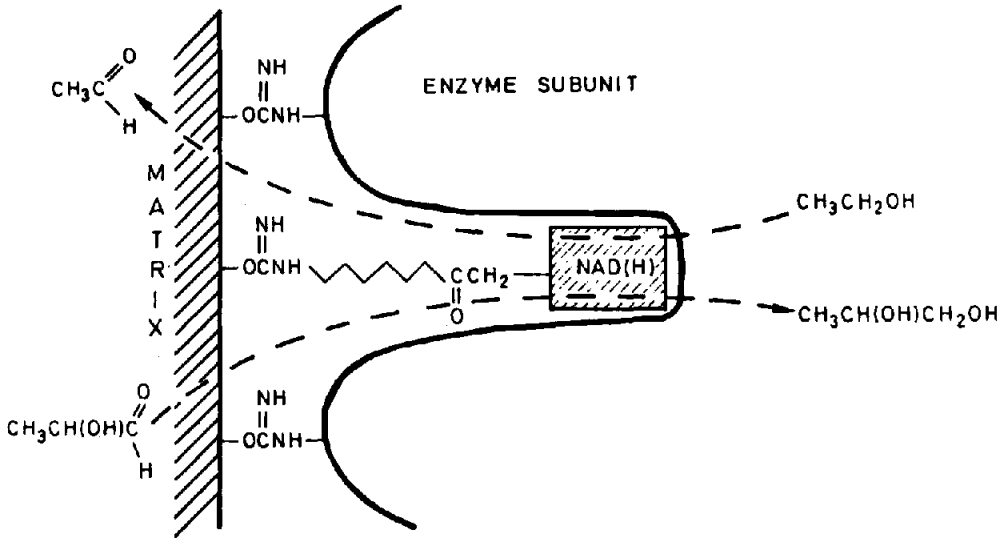


Fig.12. Schematic drawing of the active alcohol dehydrogenase/NAD(H)/Sepharose complex.

In closing I would like to stress again that this essay is a highly personal account of the area and quite some emphasis (for obvious reasons in particular regarding figure material) has been put on our own work. Needless to say outstanding research is carried out all over the world in many laboratories. It is my hope to have been able to forward some useful information to the reader of our common field of interest.

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