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GENERAL PRINCIPLES OF ENZYME STABILIZATION

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INTRODUCTION

Application of Biocatalysis: The Situation Today

Enzymes as biocatalysts differ from chemical catalysts in that they possess an extremely high catalytic activity under mild conditions and unique substrate specificity (1,2), properties that should ensure their extensive use as catalytic and regulatory elements in various areas of science, chemical technology, medicine, etc. But this has not been the case until recently because enzymes are difficult to produce (and hence costly), very labile, and hard to separate from reaction products. However, the first handicap was almost entirely done away with when techniques were developed for producing enzymes from microorganisms (3–5), and there is no doubt that the cost of production will be further reducing as microbiological technology becomes more sophisticated. Second, on the boundaries of certain chemical and biological disciplines a new line of research has emerged (6): production and investigation of immobilized enzymes (5–9). As a result of immobilization (e.g., covalent attachment of enzymes to water-insoluble particles, entrapment of enzymes into gels, adsorption of enzymes on supports), the heterogeneous catalyst can now be separated from soluble products and reused. In addition, the stability of immobilized enzymes can sometimes be significantly increased.

Because of this, enzymes have suddenly gained credence in analytical chemistry (9,10), medicine (9,11), and especially in fine organic synthesis of drugs and important biochemical compounds. A number of such enzymatic processes have been developed on a laboratory scale. One of the examples is optical resolution of racemic amino acids (12,13), synthesis of amino acids (13–15) and antibiotics (15–17), transformation of steroids (18,19),

synthesis of *N*-oxides of drugs (20,21), synthesis of pyrroleporphobilinogen (9,22), synthesis of hydroxynitriles (23), preparation of pharmaceutically pure radioactive-labeled amino acids (24), synthesis of important biochemical substances such as ATP (25), ADP (26), and others (27), and synthesis of homopolynucleotides (28,29). There is no doubt that enzymes will in the future be used still more extensively in preparative organic synthesis (9,30,31).

Moreover, introduction of enzymes has given a great impetus to the development of important technologies, such as production of glucose by hydrolysis of starch (32) and isomerization of glucose into fructose (33), preparation of L-dopa and L-methionine (34), and decrease in the content of lactose in milk (35,36). Intensive research is under way on industrial production of glucose from cellulose with the help of cellulase (37).

Approaches have been elaborated for the preparation of enzymatic light-sensitive (38) and sound-sensitive (39) materials that can serve as chemical amplifiers of weak signals (e.g., for developing new photo- and phonographic materials).

Some ways of solving energy problems with the help of enzymes have been outlined (5,7,40).

This list of problems, solved or to be solved, is by no means complete (e.g., see Refs. 5–9,41). However, we shall concentrate on the basic reason why enzymes cannot as yet be used as catalysts in important chemical reactions, i.e., on the inactivation of enzymes in technological conditions.

Why Do Enzymes Inactivate?

The loss of the apparent catalytic activity in a reaction system can be due to, for example, washing of immobilized enzyme out of a support or to partial degradation (or deformation) of the support itself (both mechanically and microbiologically) (42,43). These and similar technological problems (43) are of special interest only, as the apparent inactivation can easily be prevented by using a different support or altering the method of enzyme immobilization.

Of greater importance are inactivation processes in which the enzyme itself (or its active center) perishes:

A. Enzyme can be "poisoned" by inhibitors, such as cations of heavy metals; to prevent this, a chelating agent, e.g., EDTA (44), should be added, in either free or immobilized (45) (more feasible) form.
B. Catalytic activity may drop as a result of oxidation of functional groups of protein; in this case, protecting agents should be introduced to the system. For protection of SH groups, sulfhydryl

compounds are employed (46). Generally, antioxidants (inhibitors of free radical reactions) can be used for this purpose—they are known to be of great help for solving many applied problems in biology and medicine (e.g., see Ref. 47). In principle, oxidative destruction of enzymes can be prevented in anaerobic conditions.

C. "Digestion" of protein catalyst by microorganisms (which are invariably present in the system as an admixture) can be avoided by immobilization if the enzyme is entrapped in such a support (e.g., a microporous one) that protects it with the help of steric hindrance (5,42,43).

D. Covalent immobilization also renders impossible various polymolecular inactivation processes of protein–protein type, such as aggregation (48) and autolysis in the case of proteolytic enzymes (5,7).

E. In continuous flow reactors, an enzyme can be inactivated as a result of removal of a cofactor from the system. But this has recently been coped with in several ways, three of which seem to us most promising: use of a membrane reactor and attachment of a cofactor to a water-soluble polymer (49), simultaneous covalent immobilization of an enzyme and a cofactor (49), and use of composite enzyme systems involving regeneration of cofactor (25,49,50).

To sum up, for inactivation mechanisms A–E there are more or less adequate methods of suppression. Therefore, in the present review we shall concentrate on

F. Inactivation of enzymes due to denaturing action of the environment. We do not see much sense in dwelling on the control of denaturation of enzymes that is due to the support (51); it is much easier to choose a better support. We shall discuss the following denaturing factors: elevated temperature, unfavorable pH, and adverse effect of organic solvents.

Should Denaturation of Enzymes be Suppressed?

Prevention of the denaturation of enzymes is frequently indispensable to their technological application. Let us consider a few examples:

1. Enzymes isolated from their *in vivo* environment usually become labile and their lifetime sometimes does not exceed minutes (52–55). For example, the stability of immobilized aspartase proved insufficient for technological use of the enzyme (56).

2. For many processes, elevated temperature is desirable. This is because, first, the rates of chemical reactions, including enzymatic, increase with temperature. At higher temperatures, a required degree of conversion of the substrate in a chemical reactor will be achieved within a shorter time or with a lower amount of immobilized enzyme. This is very important, as for some technological processes the cost of the enzyme is a limiting factor, e.g., glucoamylase (32). Second, higher temperatures allow germ-free conditions to be maintained (43), which are indispensable for, say, the food industry (35,57). On the other hand, at elevated temperatures denaturation of biocatalysts intensifies (52–54).
3. Equilibrium in many technological processes is such that the required products may be obtained only if the reaction is carried out in an aqueous–organic mixture with a high proportion of the organic component. On the other hand, enzymes in such conditions as a rule lose their catalytic activity or specificity (53,58; see also Ref. 59 and references therein).
4. Sometimes the pH optimum of an enzymatic reaction and the pH range within which the enzyme is stable do not coincide. A good example is synthesis of penicillin antibiotics under the action of penicillin amidase: equilibrium in the synthesis is shifted toward the product in an acidic medium where the enzyme is rather unstable (60).
5. Enzymes are usually dealt with some time (or much) later after they are prepared, hence the need for their stabilization against inactivation during prolonged storage (61).

How to Solve the Problem?

To cope with the problem of inactivation of enzymes, several approaches have been suggested.

First, intact (or partially degraded) cells can be used (4–9); thereby the enzyme remains in its natural environment. For instance, because of the low stability of free aspartase, continuous production of L-aspartic acid involves the use of immobilized cells of *Escherichia coli* (56). Partially degraded cells retain catalytic properties even in organic solvents (62).

Second, an enzyme can be isolated from highly stable (e.g., thermophilic) strains (54,63).

But each of these approaches has a drawback of its own. The use of intact cells is hampered by diffusion of substrates and reaction products through cell walls; furthermore, metabolic pathways other than necessary

for the required catalyzed reaction can lead to the formation of undesirable side products, especially in the production of drugs. Besides, in this case the choice of immobilization methods is limited.

With enzymes from thermophilic organisms it is always troublesome (and sometimes difficult) to screen specific strains for a given catalysis.

Therefore, in this review we confine ourselves to the cases when the enzyme molecule (globule) is stabilized against denaturing actions in an artificial way.

The problem of enzyme stabilization could not have been solved merely by their immobilization, because stability of enzymes against inactivating actions sometimes increases, sometimes decreases, and sometimes remains unaltered (6). Therefore, highly stable enzyme preparations are the exception rather than the rule (7,42). Moreover, in the last year's 1000-page volume on immobilization of enzymes (9), this problem is barely touched on.

STABILITY AND STABILIZATION OF ENZYMES: DEFINITION AND GENERAL REMARKS

1. Stability of an enzyme is defined as its ability to retain the catalytic activity under the conditions when inactivation mechanisms A–E do not operate. The quantiative criterion for the stability of a given enzyme is the effective value of the first-order rate constant characterizing a monomolecular process of inactivation:

$$k_{\text{inact}} = (\mathrm{d}[E]/\mathrm{d}t)/[E]$$

2. We assume as a measure of stabilization a change (decrease) in this value induced by this or that action which the enzyme undergoes (e.g., modification or immobilization).

3. In certain systems there is apparent stabilization of immobilized enzyme compared to its native precursor, which is due, for example, to diffusion hindrances for the enzymatic reaction arising under the influence of a support (64). We shall consider only the papers where such complications are excluded; to reveal diffusion effects, we shall use the kinetic criteria formulated by Berezin et al. (65) (see also Ref. 66).

4. The problem of stabilization of enzymes against denaturing action not only has practical aspects (discussed above) it also has a theoretical significance for enzymology: attempts at stabilizing enzymes can in certain cases provide new approaches to studying the mechanisms of their denaturation. Moreover, certain stabilized enzyme preparations are models of membrane systems functioning in vivo. All these aspects will be considered in the present review.

5. We shall analyze only those methods (mechanisms) of stabilization which, being to this or that degree general, do not considerably decrease the initial level of the catalytic activity of enzymes removed from the natural environment (as a rule, not more than 2 or 3 times) and ensure their long-term functioning under unfavorable environmental conditions.

6. We shall discuss separately preparation of *water-insoluble* (truly immobilized) heterogeneous catalysts of increased stability, and of stabilized *water-soluble* enzyme preparations. The demand for soluble enzyme has been steadily increasing over the last years (5,67), foremost, in medicine (5,9,11,68) and in the processes involving water-insoluble substrates (69). An example of this is a very promising cleavage of cellulose to glucose by cellulase (37).

7. We by no means intend to discuss all the works dealing with the effect of enzyme stabilization; the number of such publications is so great that it would be impossible even to mention them in a review. We shall concentrate only on such studies where there is a physicochemical interpretation of experimental data, and such studies are not numerous.

The objective of this review, as we see it, is to formulate and to exemplify the physicochemical principles of the problem of enzyme stabilization. We shall proceed from the assumption that enzymes inactivate via the same mechanisms (see above), both in technological conditions and during storage. On the other hand, in the literature the stability of enzymes under these two sets of conditions is usually discussed separately. This is justified by the fact that the share of each inactivation mechanism can be different in each specific case. For example, at high temperature (in technological processes) denaturation (unfolding) of protein and its oxidation are mostly at work, whereas during storage (usually at low temperatures) destruction of enzymes under the action of microorganisms and proteases (especially in a humid state) should be expected. This means that for a would-be stabilization procedure to be scientifically grounded the causes of inactivation (or, even better, but not obligatory, the *mechanism* of inactivation) must be known.

STABILIZATION OF ENZYMES AGAINST THERMOINACTIVATION DUE TO UNFOLDING OF PROTEIN GLOBULE

Modification of enzymes by chemical reagents or their attachment to a water-insoluble support often entails alteration (increase or decrease) in their thermostability. This effect is often accounted for by the following phenomena (e.g., see Refs. 5–7,42,70):

1. Change in the conformation of an enzyme molecule compared to its

native structure. It is assumed that the thermostability of a protein globule depends on its conformational state.

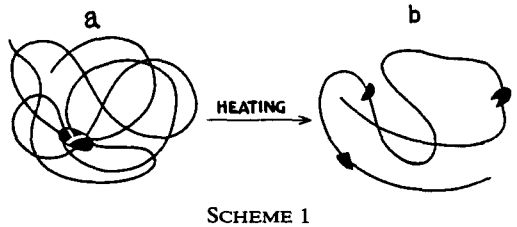
2. Change in the microenvironment of an enzyme molecule. It is assumed that the microenvironment of a protein globule affects its intramolecular linkages maintaining its native structure.

These views, even if entirely true, cannot be the foundation for elaborating general methods of stabilization of enzymes. We do not know how the stability of an enzyme is associated with its conformation and microenvironment; moreover, if this association exists at all, it should, in all probability, be unique for each enzyme. By the way, as was recently demonstrated by direct physical methods on binding between proteins and supports, the protein macromolecules undergo hardly any conformational change (71–76).

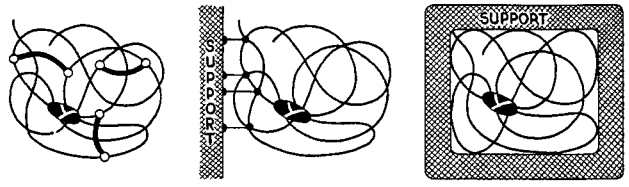
Another reason why it is difficult to outline the general ways of increasing enzyme stability is that the mechanisms of inactivation are still obscure (52,55), although intensive studies in this area have been carried out for decades now. It is likely that no general mechanism of denaturation of proteins exists (77). But it is beyond doubt that inactivation of enzymes under the action of, say, heating or denaturing agents entails significant conformational changes in protein molecules, that is, their unfolding (52–55,77) (Scheme 1). Hence the thermostability of enzymes can be increased in one more way:

3. By *rigidifying* (fixing) the native conformation of the protein globule. In fact, if unfolding is recognized as being an indispensable step of enzyme inactivation, then it is clear that the more firmly the protein globule of the enzyme is fixed, the more difficult it is to unfold it and consequently to inactivate its catalytic center. By the way, this phenomenon (variation of the rigidity of proteins) is very "popular" in nature—it is involved in the adaptation of organisms to environmental conditions (54).

An enzyme globule can be fixed to prevent its unfolding by several methods: by applying intramolecular crosslinkages (Scheme 2a), by covalent



SCHEME 1



SCHEME 2

or noncovalent attachment to support (Scheme 2b, c), and by mechanical entrappment into "tight" pores of an inert support (Scheme 2d). Let us analyze each of these approaches.

Intramolecular Crosslinkages

The literature contains abundant data on the methods of chemical modification of enzymes and on the properties of the resulting preparations. But in no case can one predict how the thermostability of enzyme will change as a result of its modification. This is associated with an extreme complexity of the molecular structure of biocatalysts (which, by the way, is still unknown for many enzymes). A good example of a rather complex dependence of the thermostability of the enzyme on the degree of modification of its surface layer is the behavior of chymotrypsin with alkylated NH_2 groups (105). The enzyme was modified by treatment with acrolein followed by reduction of Schiff's bases by sodium borohydride; the procedures were described by Feeney et al. (106). As a result, the enzyme retained almost all of its activity. It is obvious from Fig. 1 that alkylation of the NH_2 groups that were the first to undergo modification did not affect the thermostability of the enzyme. It seems that the decisive factor is the modification of the least reactive (not readily accessible but buried in the globule) NH_2 groups: the thermostability of the enzyme at the final step of its modification drastically increases (by 2 orders of magnitude). However, during subsequent alkylation (as a result of complete modification of all NH_2 groups titrated with picryl sulfonic acid) the thermostability of the enzyme preparation sharply decreases. As is seen in Fig. 1, the turning point of the effect (a sharp increase in the thermostability of the enzyme followed by its dramatic decrease) comes when only two or three out of 15 titrated NH_2 groups have been modified.

The situation is different if an enzyme is modified by bifunctional reagents which crosslink the protein globule in a covalent fashion; here the conformational stability will unfailingly increase (78).

Intramolecular Crosslinkages in Nature. Intramolecular crosslinking is widely used in nature for rigidifying (and hence stabilizing) protein

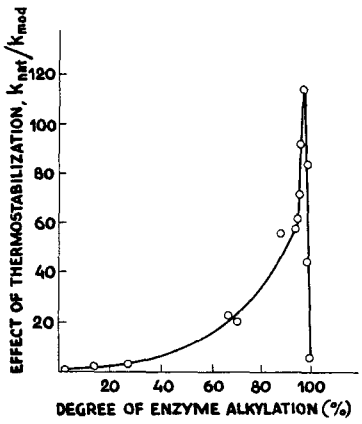


FIG. 1. First-order rate constant (k_{mod}) for monomolecular thermoinactivation of α-chymotrypsin depending on the degree (titration data) of alkylation of its NH_2 groups by acrolein (with Schiff's bases being reduced by sodium borohydride). The value of k_{nat} obtained for the native (unmodified) enzyme is assumed to be unity. Conditions: 50°C, pH 8 (5×10^{-3} M tris-HCl), 0.1 MKCl. From Martinek et al. (105).

molecules (54). These crosslinkages can be either covalent, e.g., disulfide bonds (79–81), or weak, e.g., salt bridges (82) or Ca^{2+} ions incorporated into protein molecule (83).

Artificial Crosslinking. Artificial crosslinking in protein molecules can be achieved by treating them with bifunctional reagents (84,85). The reagents commonly used are dialdehydes, diimidoesters, diisocyanates, bisdiazonium salts etc. Analysis of the literature (67,78,86–94) shows that in most cases the effects of stabilization are not high; sometimes they are due to simple (single-point) chemical modification of the enzyme (see Ref. 67) rather than to crosslinking; this was the case with glutardialdehyde (92). Sometimes the effects of stabilization proved to be more pronounced (78,91); thereby the increase in the stability correlates with greater rigidity of protein molecule (78).

It seems that when an attempt is made to enhance the stability of enzyme by treating it with bifunctional reagents, success or failure depends on whether the length of a molecule of the "bracket" corresponds to the distance between the possible centers of attachment on the protein globule. Apparently, for any protein, there is an optimal size of the intramolecular crosslinking agent. Therefore, studies on selecting the optimal size of bifunctional agents are of greatest interest.

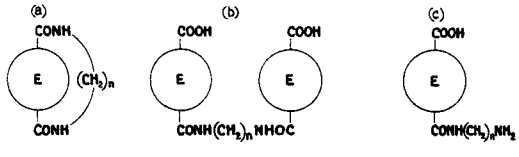
Variation in the Length of the Intramolecular Crosslinkage. Studies on intramolecular crosslinkage length are rather rare, the reason being that many of the bifunctional agents that have been used for applying brackets

are not commercially available and are rather difficult to synthesize. That is why it is very tempting to use bifunctional agents that are not suitable for intramolecular crosslinking of enzymes but become suitable after an enzyme is modified in a certain way. This was the case with some aliphatic diamines (95), as they are commercially available (the length of the hydrocarbon chain is from 0 to 12 methylene groups) and relatively inexpensive—these two factors are most important for large-scale application. Diamines can be used for crosslinking of the carboxyl groups of the enzymes (there are many of them in proteins), preliminarily activated by carbodiimide. As a result, between the amino groups of the modifier (diamine) and carboxyl group of protein a strong amide bond is formed (96) (Scheme 3).

In conditions where intermolecular crosslinking (resulting in the formation of oligomers of the enzyme; see Scheme 3b) is absent, the thermostability of an enzyme crosslinked in an intramolecular fashion considerably increases. This was the case with α-chymotrypsin, when the thermoinactivation rate constant decreased more than fortyfold compared to that of a native preparation. This stabilization effect cannot be accounted for by a simple (single-point) modification of the enzyme (Scheme 3c), as modification of chymotrypsin by a monoamine (if the enzyme was preactivated by carbodiimide)—to be more exact, by 1-aminopropanol-3—even decreases its thermostability. The effect of stabilization depends on the number of methylene groups (n) in the molecule of the modifier, $NH_2(CH_2)_nNH_2$. Figure 2A shows that the dependence of the first order rate constant for thermoinactivation of chymotrypsin crosslinked by various diamines has a sharp minimum at $n = 4$, although at $n = 2, 5, 6$ the enzyme is also stabilized.

The fact that treatment by tetramethylene diamine induces the greatest stabilization of the enzyme seems to indicate that the length of this agent best fits the distance between the carboxyl groups of the protein molecule; this, in turn, gives rise to a greater number of intramolecular linkages than with other diamines.

Ability of Enzyme to Interact with Bifunctional (Polyfunctional) Reagents. It should be expected that the more brackets are imposed in a protein molecule, the more stable it will be against unfolding and, as a consequence, against inactivation. The number of brackets is, in turn,



SCHEME 3

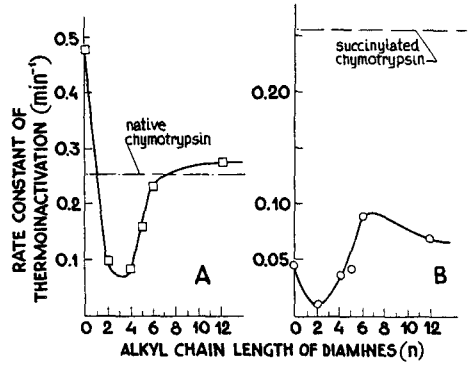


FIG. 2. First-order rate constant for monomolecular thermoinactivation of α-chymotrypsin (A) and succinylated α-chymotrypsin (B) intramolecularly crosslinked with diamines, $NH_2(CH_2)_nNH_2$. The broken line shows the level of thermostability of the native and succinylated enzymes. Conditions: 50°C, pH 7 (0.02 M phosphate). From Torchilin et al. (95).

determined by the quantity (and relative position) of the functional groups on the surface of the protein globule interacting with the crosslinking agent. It is clear that the number of the functional groups (and hence the quantity of brackets) can be increased by a respective premodification of protein.

For this purpose, Reiner et al. (97) suggest that SH groups should be introduced (with the help of *N*-acetylhomocysteine thiolactone); the bifunctional reagents were bis-iodacetamides of different aliphatic diamines.

In the case of the system described above (Fig. 2A), the enzyme was modified by succinic anhydride for the number of carboxyl groups in its surface layer to be increased (98). Succinylated chymotrypsin was treated like the native enzyme, by diamines of different length. Figure 2B shows the dependence obtained thereby (95): first, the maximal stabilization effect of the enzyme is higher; second, the maximum stabilizing effect is produced not by tetramethylenediamine (cf. Fig. 2A) but rather by a shorter bifunctional reagent, ethylenediamine. The latter fact points to a greater "populatedness" of the surface of succinylayed chymotrypsin molecule (compared to native chymotrypsin) with carboxyl groups. Thus premodification of the enzyme allows regulation of the effect of stabilization, with respect to both its magnitude and the optimal length of the crosslinkage to be achieved.

Application of Intramolecular Crosslinkages in Fundamental Biochemical Studies. Studies on the optimal size of the crosslinking agent are important not only from the practical point of view (for obtaining stabilized enzyme preparations), they also can furnish new information about the tertiary structure of proteins in solution (84) (e.g., for estimating the distance

between the functional groups in the surface layer of the protein globule) (99), about the topography of the active center (see references for Ref. 85), about the symmetry of the localization of subunits in oligomeric proteins (100,101); also see references for Ref. 85, and about allosteric and cooperative effects (102), etc. An example of fruitful application of this approach is the study of the structure of some ATPases (103,104).

Multipoint Covalent Attachment of the Enzyme to the Complementary Surface of the Support

The methods of covalent attachment of enzymes to various supports are described in detail in a number of reviews (e.g., see Refs. 5–9,11). Such method of immobilization (Scheme 2b) can, in principle, increase the conformational stability of the protein molecule and hence prevent it from unfolding and inactivation. This is evidenced by the following experimental data. First, when a study was made of the conformational mobility of immobilized proteins (with the use of a number of physical methods) it was found that, as a result of protein–support binding, the molecular structure of the former becomes more rigid (frozen) (71,72,74). The same conclusion, i.e., that after attachment to a support conformational transitions in proteins are hindered, follows from abundant indirect data (obtained by both physical methods and enzymatic activity measurements) (72,74,107–109). Second, Gabel in his pioneering work (110) showed that the greater the number of linkages by which trypsin is attached to Sephadex, the more stable is the protein against inactivation, i.e., unfolding (111), by urea.

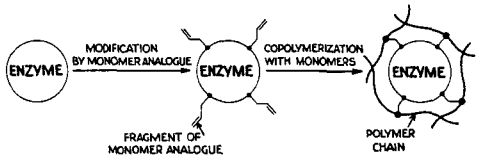
So, all the above considerations give grounds for believing (114) that the general principle of stabilization of enzymes is that of *multipoint* binding of the catalyst molecule to the support. This binding serves to make the conformation of the molecule more rigid (without necessarily altering it), hence more stable against unfolding and, ultimately, inactivation.

But this principle is difficult to realize methodologically for steric reasons as both the surface of the support and that of the protein have reliefs of their own that are, generally speaking, not congruent. In addition, it is obvious that even in the case of a multipoint interaction, when an enzyme is attached to the support with only a small portion of its surface, one should hardly expect that all of its molecule will be rigidified. This seems to have been overlooked by some authors whose attempts to stabilize enzymes were unsuccessful (e.g., see Refs. 71,112,113). The problem is actually that of providing a support with a surface *complementary* to that of the enzyme molecule; only then can the multipoint protein–support interaction favorable for stabilization of the enzyme be realized.

Preparation of a Polymeric Support Complementary to the Molecule of Immobilized Enzyme. The gist of our approach (114–116) to preparation of a polymeric support is the following: the enzyme is modified by an analogue of a monomer and the resulting preparation is copolymerized with the monomer proper. We now have an enzyme chemically incorporated in the three-dimensional lattice of the polymer gel, the points of the enzyme–support binding being the centers of premodification of the enzyme molecule (Scheme 4). It is obvious that, because of the principle on which the method is based, the microsurface of the gel around the entrapped protein molecule must be complementary to the surface of the latter.

The method for preparation of copolymerization-immobilized enzymes was elaborated by Jaworek et al. (117). Their goal was to prevent immobilized enzymes from being washed out from gel supports. We have independently suggested this method (114–116) for stabilization of enzymes. As analogues of the monomer, one may use acylating and alkylating agents with a double bond capable of copolymerizing, for example, acryloylchloride, which acylates NH_2 and OH groups of protein, or acrolein (114), which interacts with NH_2 groups; if Schiff's bases are reduced by sodium borohydride, protein becomes alkylated. There is also a method of vinylation of the carboxyl groups of proteins (118). Acrylamide, sodium methacrylate, and 2-hydroxyethylmethacrylate can be used as comonomers and *N,N'*-methylene-bisacrylamide as a crosslinking agent. All these reagents are available and inexpensive.

The Scale of the Stabilization Effect. A good example of the scale of the stabilization effect is Fig. 3, where the data on the rate of inactivation of α-chymotrypsin entrapped in polymethacrylate and polyacrylamide gel are presented in Arrhenius coordinates (114). Thermostability of immobilized enzyme preparations is so much higher than that of the native enzyme that they are impossible to compare experimentally, i.e., at the same temperature. One has to resort to comparison of the values of thermoinactivation rate constants determined by extrapolation, e.g., at 60°C (the vertical broken line in Fig. 3). At 60°C acryloylated chymotrypsin entrapped in methacrylate and polyacrylamide gels is 1000 and 200 times as stable as the native enzyme. The difference is still more striking if comparison is made with free acryloylated chymotrypsin.



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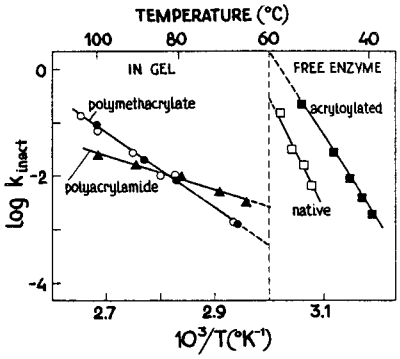


FIG. 3. Temperature dependence of the first-order rate constant (min^{-1}) for monomolecular thermoinactivation of free (native and acryloylated) and immobilized (chemically entrapped in polymeric gel) α-chymotrypsin. Conditions: pH 8 (0.005 M tris-HCl), 3 M KCl. ●, Water-insoluble enzyme; ○, water-soluble preparation (see text). From Martinek et al. (114).

Figure 3 shows that the effective values of activation energies of thermoinactivation for acryloylated chymotrypsin entrapped in both polymethacrylate and polyacrylamide gels (35 and 15 kcal/mole, respectively) are much lower than that of the native enzyme (110 kcal/mole). This means that, as the temperature rises, the effect of stabilization of the immobilized enzyme will increase compared to the native enzyme. For example, extrapolation in Fig. 3 shows that at 102°C the gel-entrapped enzyme is 10^8 times as stable as the native enzyme.

Dependence of Stabilization Effect on the Number of Linkages Between Immobilized Enzyme and Support. It is noteworthy that for systems analyzed above (Fig. 3) the effect of stabilization does not depend on the density of the gel support (that is, on the concentration of the polymer or, to be more exact, on the concentration of the monomer in the polymerization mixture over the range of 10–50% wt/wt) or on the degree of its swelling (which depends on the quantity of the crosslinking agents, i.e., *N,N'*-methylenebisacrylamide). Moreover, if copolymerization of acryloylated derivative of the enzyme with comonomers is performed without the crosslinking agent, the resulting gellike polymer can be *completely dissolved in water* and, most important, the resulting enzyme preparation has a thermostability as high as that of the enzyme immobilized in a water-insoluble manner (Fig. 3) (114). This seems to be due to the fact that the polymeric structure of the gel in the vicinity of the enzyme molecule, being congruent to its surface, is primarily determined by the number of the crosslinkages with protein.

The thermostability of copolymerized enzyme is, in turn, affected by the degree of its covalent binding with the support; that is, the greater the number of linkages between the immobilized enzyme and support, the

greater is the thermostability of the catalytic activity in the system. For example, Fig. 4 shows that the effective thermoinactivation rate constant of α-chymotrypsin (at 60°C) decreases more than a thousandfold if the degree of attachment of the enzyme to polymethacrylate gel is increased (114).

Mechanism of Stabilization. The effect of stabilization cannot be ascribed to alteration of the thermostability of the enzyme resulting from its having been chemically modified; for example, free acryloylated chymotrypsin is even less thermostable than the native enzyme (see Fig. 3).

Neither can the stabilization be attributed to the effect of the gel microenvironment on the enzyme, as the thermostability of chymotrypsin, physically entrapped in polymethacrylate gel of the same concentration, is practically the same as that of the free enzyme (119).

The nature of the gel is actually not decisive in enzyme stabilization. Increase in the thermostability was observed if the enzyme immobilized by copolymerization was chemically entrapped into polyelectrolyte, polymethacrylate (114), and electroneutral polyacrylamide (114) or poly(2-hydroxyethyl)methacrylate (120) gels (e.g., see Fig. 3).

The effect of stabilization occurs with various kinds of premodification, i.e., alkylation of NH_2 groups by acrolein or acylation of protein with acryloyl chloride (114) or vinylation of the carboxyl groups (118).

Finally, stabilization was achieved with various enzymes, i.e., α-chymotrypsin and trypsin (114), glucose oxidase (120), and penicillin amidase (121).

All this means that the key role belongs to the entrapment of the enzyme molecule into the three-dimensional lattice of the gel rather than to the premodification method or the nature of the gel. When the protein is fixed, the unfolding of the globule on heating (Scheme 1) is more difficult. It should be emphasized that the native (catalytically active) conformation of

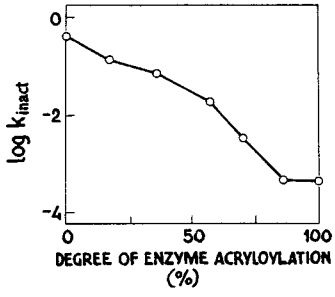
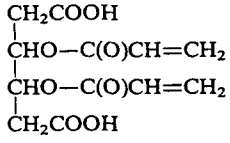


FIG. 4. Dependence of the first-order rate constant (min^{-1}) characterizing the monomolecular thermoinactivation of α-chymotrypsin entrapped in polymethacrylate gel on the degree of the enzyme–support covalent binding. Conditions: pH (0.005 M tris-HCl), 3 M KCl, 60°C. From Martinek et al. (114).

the active center can be maintained only if the enzyme is attached to the support in a multipoint fashion (Fig. 4).

Perspectives. It is beyond doubt that the thermostability of a copolymerized enzyme should depend (at a given number of crosslinkages between the protein and the support) on the rigidity (flexibility) of the polymer chain covalently attached to the protein globule and maintaining its native structure; that is, with more rigid (less flexible) polymers (with respect to molecular level) one should expect greater stabilization effects (115). This principle should be verified experimentally with the help of, for example, protein modifiers and comonomers containing at least two polymerization centers in a molecule and thus ensuring formation of a branched (heavily crosslinked) polymeric structure of the gel. These compounds should be sufficiently soluble; this means that they should be electrolytes, e.g.,



which can be synthesized by acylating tartaric acid with acryloyl chloride. Besides, the polyelectrolytic character of the polymer should make its chains still more rigid (see Ref. 122).

Gels used as supports have some drawbacks (123), e.g., low mechanical strength of gel granules. These disadvantages can be coped with if polymerization is performed in the macropores of sufficiently strong carriers, e.g., inorganic ones, which has already proved feasible (32,124,125).

Covalent attachment of enzymes to elastic supports (including gellike ones) allows the catalytic activity to be regulated mechanically (126). With polyelectrolytes as supports the catalytic activity of immobilized enzymes can also be regulated by changing the ionic strength of the solution (127). These regulatory aspects can prove useful in organic synthesis involving enzymes (in chemical technology).

Fundamental Studies. The use of covalently immobilized enzymes in fundamental studies is described in detail in Mosbach (9).

Multipoint Noncovalent Interaction of Enzyme with Support

Multipoint interaction of an enzyme with a support can in principle ensure the stabilization of a biocatalyst even if it has been immobilized by physical methods. Physical methods of immobilization [e.g., mechanical entrapment in gels (123), microcapsulation (128), and adsorption on supports (129,130)] have been studied rather comprehensively (see Refs.

5,9,11). It is only recently, however, that the thermodynamic causes (the mechanism) of enzyme thermostabilization have been discussed and recommendations have been given for preparation of stabilized enzymes (119).

Scale of the Stabilization Effect. By way of example let us discuss in greater detail the thermostability of α-chymotrypsin mechanically entrapped in polymethacrylate gel. With this polyelectrolyte as support, the enzyme can form a great number of weak linkages, electrostatic or hydrogen.

Figure 5A shows the dependence of the logarithm of the effective rate constant of thermoinactivation of chymotrypsin on the concentration of polymethacrylate gel (up to the limit of solubility of the monomer in water). One can see that if the concentration of the gel in water changes from 0 to 30% wt/wt, the rate of inactivation (at 60°C) of the enzyme is almost constant but sharply drops if the concentration of the gel is further increased. With the gel concentration of 50% wt/wt, the scope of the stabilization effect amounts to 10^5 times. Moreover, by extrapolating the straight line of the Arrhenius dependence ($\log k_{inact}$ vs. $1/T$) it was found (119) that the lifetime of the enzyme immobilized in 45% wt/wt polymethacrylate gel is to be hundreds of millions of years.

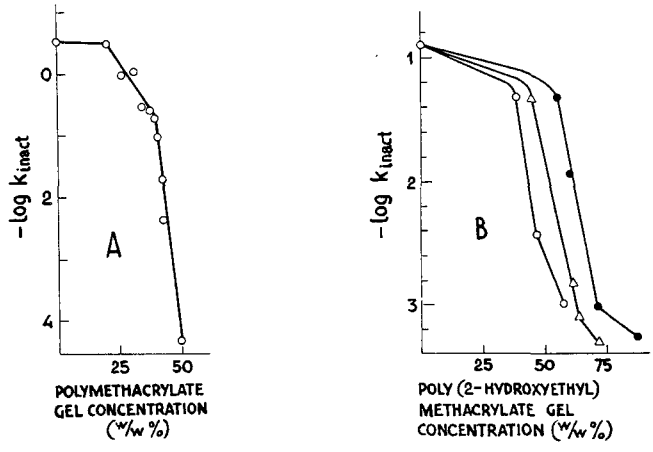
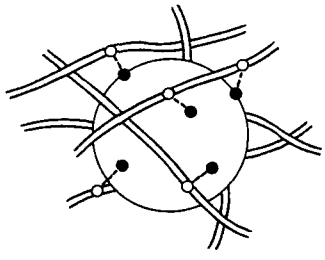


FIG. 5. (A) Dependence on gel concentration for the first-order rate constant (min^{-1}) characterizing the monomolecular thermoinactivation of α-chymotrypsin mechanically entrapped in polymethacrylate gel. Conditions: pH 8, 60°C. From Martinek et al. (119). (B) Dependence on gel concentration for the first-order rate constant (min^{-1}) characterizing thermoinactivation of glucose oxidase mechanically entrapped in poly(2-hydroxyethyl)methacrylate gel containing copolymerized residues of methacrylic acid (mol %): ●, 0.3; △, 0.6; ○, 4.6. Conditions: 65°C, pH 5.8 (0.2 M acetate), 1 nM EDTA. From Kulys et al. (131).

Mechanism of Stabilization. The mechanism of stabilization is conditioned by the interaction of enzyme molecules with polymeric support. In principle, protein molecules can form electrostatic and hydrogen bonds with carboxyl groups of the support, i.e., of polymethacrylate. However, these bonds are relatively weak and can hardly be realized in a solution of the polymer or in diluted gels, where formation of such a complex should involve great entropy losses for "quenching" the transitional and rotational movements of the molecules of the enzyme. The situation is different in concentrated gel: there the transitional, and possibly rotational, movement of protein molecules is to a great extent decelerated (independently of the complex formation) because of steric hindrances formed by the dense three-dimensional lattice of the polymer. This means that, in concentrated gel, formation of the protein–support complex should be thermodynamically more favorable, as there are no (or much less) free energy losses due to the transitional or rotational entropy of the enzyme globule on its being adsorbed on the polymeric support. It is not excluded that in concentrated gel the mobility of the polymer chains is also somewhat limited; hence, compared to diluted gels (or polymer solution), the entropy expenditure of free energy necessary for the support to be "frozen" (when its complex with the enzyme is formed) is much lower. Finally, with concentrated gel it should be expected that enzyme–support interaction will be multipoint, at least for purely steric reasons, as the polymer chains adhere to the enzyme globule from all the sides (see Scheme 5). And, as has been exemplified above with covalent enzyme–support binding, such multipoint interaction may lead to a sharp increase in the thermostability of an immobilized enzyme.

All these conclusions were drawn (119) from investigation of the thermostability and transitional and rotational mobility of enzyme molecules depending on the concentration of gel.

Other Systems Where Enzyme is Stabilized by Mechanical Entrapment into a Polymer Gel. The above mechanism of stabilization of enzymes mechanically entrapped in concentrated polymer gels (forced cessation of



SCHEME 5

the transitional movement of the protein molecule followed by multipoint interaction of the protein with relatively rigid polymer chains) is operative with supports other than polymethacrylate and enzymes other than chymotrypsin. For example, Kulys et al. (131) have recently reported a sharp increase in the thermostability of an enzyme with a tertiary structure, glucose oxidase, on its being entrapped mechanically into poly(2-hydroxyethyl)methacrylate gel. As shown in Fig. 5B, decrease in the rate constant of thermoinactivation of this enzyme at sufficiently high concentrations of this gel has a threshold character (similar to the previously studied system shown in Fig. 5A). With electroneutral poly(2-hydroxyethyl)methacrylate gel it should be expected that the multipoint interaction between the enzyme and support would occur as a result of formation of hydrogen bonds. It is interesting to note that when a negative charge is introduced into the gel [as a result of copolymerization of (2-hydroxyethyl)methacrylate with methacrylic acid] the stabilization can be realized at lower concentrations of the gel (see Fig. 5B). As the content of methacrylate in the support increases (Fig. 5B), the "concentration threshold of stabilization" becomes closer to that observed for the "pure" polymethacrylate gel (cf. Fig. 5A and 5B). This phenomenon seems to be due to an additional electrostatic interaction of the immobilized enzyme with the support.

The specific protein–support interaction that produces a favorable effect on the thermostability of the enzyme can be different from the systems considered above (see Figs. 5A and 5B) where electrostatic or/and hydrogen bonds are at work. Let us consider the data (132) on thermostability of acetylcholinesterase, glucose oxidase, and peroxidase mechanically entrapped into hydrophobic silicon-organic gel, silastic. The thermostability of the first enzyme drastically increased on its being entrapped into such gel, whereas the thermostability of the last two enzymes remained unchanged on immobilization. In principle, all three enzymes could have become stabilized against unfolding and hence thermoinactivation by having formed multipoint hydrophobic contacts with the support. But, as follows from the above mechanism, an enzyme–support interaction can be thermodynamically effective only if the molecular mobility of protein molecules mechanically entrapped into gel is retarded as early as in the ground state. In silastic of the given concentration, the inhibition of transitional diffusion (and hence stabilization) is likely to occur in the large molecules of acetylcholinesterase rather than relatively small molecules of glucose oxidase and peroxidase.

Perspectives. One cannot give preference to any method of physical immobilization without knowing the purpose for which a given enzyme preparation is used. If, however, one wishes only to increase the thermostability of an enzyme as a result of immobilization, the most attractive

method, in our opinion, is mechanical entrapment of an enzyme into gel capable of specific interaction with the protein. The effect can even be enhanced compared to the systems analyzed above. To this end, the structure of the gel is optimized by using more than one monomer (the crosslinking agent is not taken into account) and thus introducing into support different functional groups (123,133) (e.g., see Fig. 5B).

On the other hand, however, one should remember that the stabilization effect in such systems depends entirely on the concentration (density) of the gel (see Figs. 5A and 5B); that is, the stabilizing effect is produced (if it is produced at all) by concentrated gel. Consequently, as a result of swelling of gel particles (granules) in water, the effect of stabilization reversibly (119) disappears. This should, at first glance, limit the applicability of such systems for preparation of stabilized enzymes. However, the swelling of gel in water can be prevented (with the effect of thermostabilization being retained) if crosslinked gel is used; the crosslinking is carried out either directly in the course of polymerization (using comonomers of *N,N'*-methylene-bisacrylamide type) or to treat gel granules (size of granules dozens of micrometers) by bifunctional crosslinking agents (134).

Thermostabilized enzyme preparations based on gels will also find application in catalysis involving organic solvents, as gel particles containing an aqueous solution of an enzyme will not swell if placed in a water-immiscible organic solvent. This means that the effect of thermostabilization (of the type shown in Figs. 5A and 5B) will be preserved. Enzymatic reaction in water–organic solvent systems are described in greater detail below.

Biological Aspects (*Model of Biological Membranes*). The above mechanism of enzyme stabilization (119) mechanically entrapped in concentrated polymeric supports (i.e., induced arrest of transitional movement of an enzyme molecule followed by multipoint interaction of protein with a relatively rigid support) possibly functions in vivo in membrane enzymatic systems (135,136), where, first, the mobility of protein molecules is rather low (the transitional and rotational movements are suppressed) (137), and, second, the enzyme molecules can interact with phospholipids and structural proteins of the membrane phase to form relatively weak bonds, hydrophobic, electrostatic, hydrogen etc. (138). Moreover, such formal similarity allows one to understand certain aspects of the functioning of biological membranes. It is known that environmental temperature adaptation of organisms involves changes in the thermostability of membrane enzymes (54). The molecular mechanism of such regulation is possibly that in membranes the proportion of unsaturated lipids changes, which should affect the viscosity of the membrane phase and thus the molecular mobility of the enzyme molecules entrapped into it. In its turn, the

molecular mobility and thermal stability of enzymes mechanically entrapped in support are interconnected, as was demonstrated (119) in a model system consisting of α-chymotrypsin in polymethacrylate gel (see above).

Mechanical Entrapment of Enzymes in "Tight" Pores of Support

Protein globules can be prevented from unfolding if a molecule of a native enzyme is placed in a certain cell that does not interact with it either chemically or sorptionally but that is sufficiently "tight" to prevent formation of an unfolded conformation for steric reasons. In this case, the native conformation of protein globule could be maintained in a purely mechanical fashion (Scheme 2d). This mechanism has been more than once hypothesized in the literature (sometimes in a very incoherent fashion) (139–142), but experimental evidence for it has been furnished only recently (134,143,144) when the properties of α-chymotrypsin and trypsin in polyacrylamide gel or in/on polysaccharide supports were studied.

Polyacrylamide Gel. Polyacrylamide gel is a very suitable support in this kind of study because, first, it is chemically inert (145), second, the size of its pores can be varied by changing the concentration of the monomer (146), and, third, at sufficiently high concentrations of the gel, the size of its pores is commensurate with the sizes of enzyme globules (147,148).

Two methods were used to achieve mechanical entrapment of the enzyme into gel. Polymerization of acrylamide of a certain concentration was carried out directly in a solution of the enzyme, as described by Martinek et al. (114,119), or gel particles (taken without the enzyme and thoroughly washed from low molecular weight admixtures) were impregnated with the solution of the enzyme and then lyophilized until the required concentration of polyacrylamide was achieved (143,144). In both cases the enzyme displays the same thermostability (Fig. 6): at a moderately high concentration of the gel (less than 45% wt/wt) there is no appreciable stabilization of the enzymes against irreversible monomolecular thermoinactivation, whereas in a more dense support (more than 50% wt/wt of polyacrylamide) the thermostability of the enzyme sharply increases.

Scale of the Stabilization Effect. The thermostability of the enzyme mechanically incorporated in highly concentrated polyacrylamide gel (lyophilized powder) is so much higher than that of the enzyme in solution that these values cannot be determined experimentally at the same temperature. To find this out, we had to study the temperature dependence of the rate of monomolecular thermoinactivation; linear extrapolation of the data in Fig. 7 shows that at the midpoint of the temperature range, i.e., 120°C (vertical broken line) the effect of stabilization in fully dried gel is 10^{13} times as high.

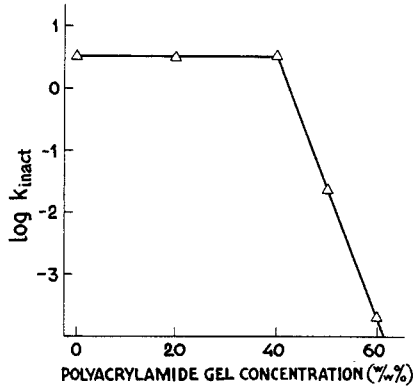


FIG. 6. Dependence on gel concentration for the first-order rate constant (min^{-1}) characterizing the monomolecular thermoinactivation of α-chymotrypsin mechanically entrapped in polyacrylamide gel. Conditions: 60°C, pH 8 (0.02 M tris-HCl), 0.1 M KCl. From Goldmacher (134) and Martinek et al. (144).

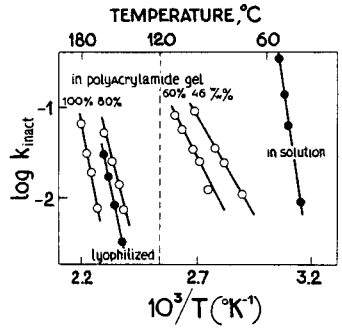


FIG. 7. Temperature dependence of the first-order rate constant (min^{-1}) characterizing the monomolecular thermoinactivation of α-chymotrypsin mechanically entrapped in 46%, 60%, 80%, and 100% wt/wt polyacrylamide gel. The concentrations of the polymer were determined after washed 60-μm gel granules had been lyophilized. For comparison the thermostabilities of the enzyme in an aqueous solution (pH 5–8, 5×10^{-3} M tris) and in a lyophilized state are given. From Goldmacher (134) and Martinek et al. (144).

For gels of lower concentration, the effect of stabilization is, of course, lower.

Mechanism of Stabilization. The mechanism of stabilization is not associated with the effect of nonpolymerized acrylamide (149), as it was completely washed from powdered gel (143,144). Neither can the stabiliza-

tion be explained by chemical attachment of the enzyme to the gel, as both chymotrypsin and trypsin could be washed completely from swollen gel (143,144). The enzymes hardly ever interact with the support in a sorptional way up to a gel concentration of 50% wt/wt (at which pronounced thermostabilization is observed). This indicates that at a low temperature (5–25°C) the protein globule retains a high rotational mobility (119,143,144).

One has then grounds for believing that as the concentration of the gel increases, the size of the pores decreases, and the enzyme globule is fixed so firmly that it cannot unfold (denature) because of the "cage effect" (Scheme 2d).

One could have assumed a different mechanism of stabilization, i.e., dehydration of protein, believing that on dehydration the mobility of the polypeptide chains becomes lower and the unfolding of the globule will be less pronounced. One cannot choose between the two possibilities having studied the thermostability of a lyophilized preparation of a free enzyme. In this state the molecules of protein seem to have a sufficiently dense packing when each globule can be entrapped in a "tight pore" of the same globules (similar to immobilization in gel). Therefore, the reason for the extremely high thermostability of dried (lyophilized) enzyme preparations (139,141,142) (e.g., chymotrypsin; see Fig. 7) can be both "dehydration of polypeptide chains" and the "cage effect." This problem was resolved when polysaccharide supports were used.

Polysaccharide Supports. Polysaccharide supports are inert and do not interact with proteins. This is evidenced by the following fact: although the transitional diffusion of protein molecules (lactoglobulin, ribonuclease, etc.) in 40% solution of dextran is almost arrested, their rotational diffusion remains almost the same (compared to an aqueous solution containing no polysaccharide) (150).

To resolve this question, the thermostability of chymotrypsin covalently bound to two polysaccharides, microcrystalline cellulose and Sephadex, has been studied (144). The two supports are not greatly different in chemical composition, but structurally they are very dissimilar—Sephadex, unlike cellulose, is porous. This means that if with cellulose the enzyme is attached to the surface then in Sephadex the protein molecule will be entrapped in pores. That is why on lyophilization in Sephadex (and not in cellulose) the "cage effect" can be realized. Table 1 shows that covalent immobilization only slightly affects the thermostability of chymotrypsin on both polysaccharide supports (compared to the free enzyme). However, another result seems to be more important; i.e., additional lyophilization of the enzyme attached to the surface of microcrystalline cellulose does not greatly affect the thermostability of the enzyme preparation (see Table 1).

TABLE 1. **First-Order Rate Constants (min^{-1}) for Irreversible Monomolecular Thermoinactivation (60°C) of Native and Immobilized α-Chymotrypsin (134,144)**

Conditions	Preparation		
	Free enzyme	Enzyme attached to the surface of microcrystalline cellulose	Enzyme chemically entrapped in pores of Sephadex
Solution or suspension at pH 8 (2×10^{-2} M tris + 0.1 M KCl)	3	0.1	0.08
In lyophilized state	0[a]	0.05	0[a]

[a]No thermoinactivation was detected for 30 min even at a higher temperature, 100°C.

The situation is entirely different in the case of the enzyme attached to Sephadex G-150; this lyophilized enzyme preparation was not thermoinactivated at all under comparable conditions.

Since the only significant difference between these polysaccharide supports is that in Sephadex the enzyme after lyophilization is firmly entrapped in a pore and in microcrystalline cellulose it is attached to the surface of the particle, one can conclude that such an enormous increase in the thermostability of the enzyme in concentrated porous supports (Sephadex, see Table 1, and polyacrylamide, see Fig. 7) should be ascribed to the "cage effect" rather than to dehydration of protein.

Perspectives. Enzyme preparations with such a high thermostability can find many applications if swelling of support in water is eliminated. This can be achieved by using highly crosslinked polymeric supports or by providing organic solvents immiscible with water for enzymatic reactions (see below).

Immobilization of protein globules in tight pores of an inert support opens up new perspectives for stabilization (owing to the cage effect) of even very labile enzymes, which usually, on being immobilized, become inactivated by interacting with the support. In nature such a mechanism (based on the "cage effect") is likely to stabilize proteins in spores against adverse effects of the environment.

Approaches Based on the pH Dependence of the Rate of Thermoinactivation

The rate of thermoinactivation sometimes greatly depends on pH (e.g., see Refs. 6,98). In this case the denaturation of the enzyme can be inhibited using the procedure based on the physicochemical principles described below.

STABILIZATION OF ENZYMES AGAINST INACTIVATION UNDER THE ACTION OF EXTREME pH VALUES

Many enzymes rapidly and irreversibly lose their catalytic activity under the action of extreme pH values (i.e., at pH values of the medium that are far from the pH range of their action) (52). A good and well-studied case in point is "alkaline" inactivation of porcine pepsin (which starts as early as at pH 5) or "acidic" inactivation of chicken lactate dehydrogenase (which is effective as early as at pH 4).

In principle, on being immobilized, the enzyme can change its pH stability (as well as its thermostability; see previous section). This has been observed experimentally with many enzymes (5,6,42,151). This is usually explained (e.g., see Ref 6) by alteration of the microenvironment of the enzyme on its immobilization; another possible explanation is alteration of the conformation of the enzyme on immobilization induced, for example, by its chemical modification or interaction with the support.

However, similarly to stabilization of the enzymes against heating, we must state that, although these considerations may be absolutely correct, they cannot be used as a foundation for elaborating the general principles of enhancement of the pH stability of the enzymes, because it is not known how the latter is associated with the microenvironment of the enzymes and their conformation. On the other hand, we can now single out several general physicochemical approaches with the help of which the pH stability of the enzyme will invariably be increased as a result of immobilization.

Rigidification (Fixation) of the Native Conformation of Enzyme Globule

As in thermoinactivation, pH inactivation of enzymes includes unfolding of the protein globule (53) caused by the alteration of the balance of electrostatic and hydrogen bonds resulting from pH-induced alteration of the ionization state of ionogenic groups of protein. If this is the case, all the recommendations which were given when discussing stabilization of enzymes against thermoinactivation (see above) should be valid.

By way of example, let us consider the data on the pH dependence of the rate of thermoinactivation of glucose oxidase (131). Figure 8 shows that the rate of inactivation of the free enzyme (temperature 56°C) sharply increases with increase in pH. As a result of mechanical entrapment of the enzyme in concentrated polymeric gel (2-hydroxyethylmethacrylate copolymerized with methacrylic acid), its thermostability considerably increases (see Fig. 5B); therefore, the pH dependence of the rate of thermoinactivation of an immobilized enzyme preparation was studied at a higher temperature, i.e., 65°C. The most interesting result obtained thereby

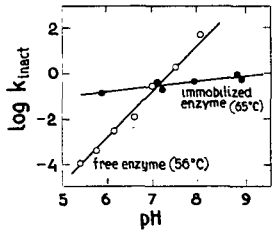


FIG. 8. pH dependence of the first-order rate constant (min^{-1}) characterizing the thermoinactivation of free glucose oxidase and glucose oxidase mechanically entrapped in polymeric gel (52% wt/wt 2-hydroxyethylmethacrylate copolymerized with 1% wt/wt of methacrylic acid). Conditions: 56°C and 65°C for the free and immobilized enzymes, respectively; 5×10^{-3} M tris-HCl or phosphate. From Kulys et al. (131).

is that the rate of thermoinactivation of this enzyme becomes insensitive to pH changes as a result of immobilization (cf. the slopes of the lines in Fig. 8). Kulys et al. (131) explained this by the fact that the polymeric support forms hydrogen and electrostatic bonds (of the type indicated in Scheme 5) with the enzyme globule which maintain rather reliably its catalytically active conformation, now so strong that the change in the ionic state of the globule (due to pH changes) is no longer important in the mechanism of its denaturation.

Immobilization of Enzymes on Support with Buffer Properties

Let us imagine that an enzyme is immobilized on a support carrying ionogenic groups possessing buffer properties of considerable capacity. Then, when pH of the bulk solution changes, the change in the micro-pH in the support (i.e. around the enzyme) will always be much less pronounced. As a result, the apparent pH stability of immobilized enzyme compared to its native precursor will increase.

This was brilliantly illustrated by Kaplan et al. (152), who studied acidic inactivation of native and covalently bound to glass lactate dehydrogenase. The native enzyme at pH 3.2 (25°C) almost totally inactives within 1 hr, whereas the immobilized enzyme under these conditions retains almost 100% activity even after 35 days of incubation (Fig. 9). The buffer properties of the surface of the support in this case are proved by the fact that the catalytic activity of the glass-attached enzyme, unlike native dehydrogenase, hardly ever depends on the pH of the medium (152).

Shift of the pH Profile of the Rate of Enzyme Inactivation

Since the classic studies of Katchalski et al. (153,154), the shift of the pH profile of the catalytic activity of enzymes on their being attached to

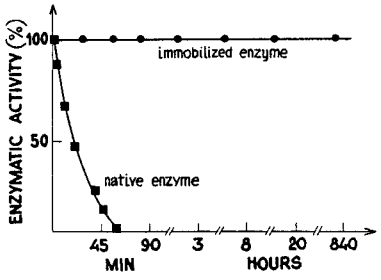


FIG. 9. Stability of native lactate dehydrogenase and lactate dehydrogenase covalently bound to porous glass (25°C, pH 3). From Dixon et al. (152).

polyelectrolytes has been an established fact (for review, see Ref. 65). On immobilization of enzymes on polyanions, the pH profile of the enzymatic activity shifts toward the alkaline region (because of high concentration of hydroxonium ions in the phase of support compared to that of the solution). On immobilization on polycations, the shift is toward the acidic region (because of a lower concentration of hydroxonium ions in the support phase compared to the solution) (e.g., see Fig. 10A). The shift of the pH profile of

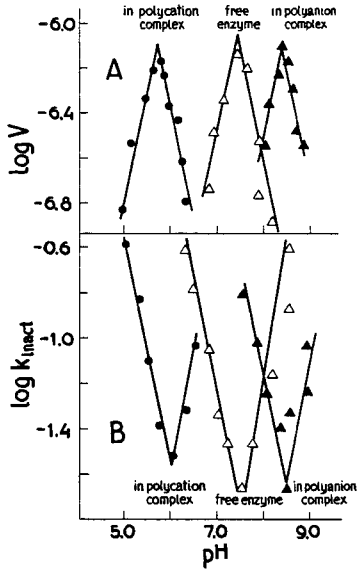


FIG 10. (A): pH dependence of the maximal rate (at 25°C) of hydrolysis of benzylpenicillin, catalyzed by penicillin amidase in the native and immobilized states. (B): pH dependence of the inactivation rate (at 50°C) of penicillin amidase in the native and immobilized states. From Streltsova et al. (155).

the catalytic activity of the enzymes on immobilization should also take place with a nonelectrolyte support because protons are distributed between the support and the medium in a certain specific way (65).

It is obvious that similar effects should take place during inactivation of the enzyme; i.e., the difference in the pH values of the solution and the support phases should induce a shift in the apparent pH profile of the inactivation rate. It was demonstrated by Katchalski et al. in his series of investigations (see review in Ref. 6) that, as was to be expected, enzymes attached to a polyanionic support have a higher stability in alkaline pH and enzymes attached to a polycationic support become more stable in acidic solutions. This phenomenon illustrates the data of Streltsova et al. (155), in which the activity and stability of penicillin amidase immobilized on different supports were studied. One can see (Fig. 10B) that the pH profiles of the rate constants of enzyme inactivation shift depending on the nature of the support, in complete agreement with the theory.

ENZYMATIC REACTIONS IN ORGANIC SOLVENTS WITH A LOW CONTENT (OR IN THE ABSENCE) OF WATER

Many enzymes have been adapted by nature for functioning primarily in aqueous solutions. This is a limitation for enzymes that are intended to be used in technological processes. Many chemical reactions are thermodynamically directed toward the desired product only in certain organic solvents. This is associated with specific solvatation effects, with the solubility of the certain components of the reaction, and, last but not least, with the fact that sometimes, in addition to the required product, water is formed. Hence in aqueous solution the equilibrium is shifted toward the starting substances. This prevents biocatalysis from being introduced into some important processes, such as synthesis of esters or amides, polymerization of amino acids and sugars, and dehydration reactions.

To overcome this difficulty, water should be replaced by a nonaqueous solvent as a reaction medium. This idea has been developed in a great number of works and, there have been attempts at carrying out enzymatic reactions in organic solvents or aqueous–organic mixtures with a high concentration of the nonaqueous component (58,156–181). But all the authors stated that when water is replaced by an organic solvent as a reaction medium, the catalytic activity of enzymes drastically decreases and their substrate specificity disappears.

A partial solution to the problem is to use an organic component which produces the lowest denaturing effect on the enzyme (181). Two other approaches seem, however, to be promising, i.e., the use of biphasic

aqueous–organic systems (mixtures of water with water-immiscible organic solvents) (59,182) and entrapment of enzymes in "reversed" surfactant micelles (183). Let us consider in more detail the physicochemical principles of these three approaches.

Criterion for Selecting the Optimal Organic Solvent

To carry out enzymatic processes in nonaqueous or aqueous–organic solvents with a high content of nonaqueous component, use is made of an organic solvent such as dimethylsulfoxide (158–165), formamide (163,166), dimethylformamide (163,167–169), dioxane (170–174), ethylene glycol (172,175), glycerol (175,176), alcanols (165,172,177–180), or acetone (165–174). The choice of the solvent is usual incidental.

The criterion for selecting the optimal (from the point of view of its effect on the enzyme) solvent can be evolved from consideration of the forces involved in the maintenance of the native structure of the protein molecule in aqueous solution—the dominant role here belongs to hydrophobic interaction (79). Consequently, a nonaqueous solution which is "good" for the protein should not destroy such interactions. Which solvents possess such a property?

To answer this question, let us turn to the data on the model process of formation of surfactant micelles in various solvents. Ray (184) concluded that hydrophobic interaction is just one of the kinds of solvophobic interactions. In terms of solvophobic interactions, all solvents can be divided into three classes. The first class comprises water, glycerol, ethylene glycol, aminoethanol, formamide, etc.; the second class comprises methylformamide and dimethylformamide; the third class comprises methanol, ethanol, toluene. Solvophobic interactions are inherent to the greatest degree in the solvents belonging to the first class. For example, Ray (184) found that surfactant micelles form only in them. To a much lesser degree, solvophobic interactions can be realized in the solvents of the second class and are practically nonexistent in the solvents of the third class. Ray (184) attributes this difference to the fact that the solvents of the first class contain at least two or three fragments in their molecule capable of forming hydrogen bonds. As a result of intermolecular interactions, hydrogen bonds form rigid (thermodynamically unfavorable) structures around solvophobic dissolved molecules. Among the solvents of the first class, solvophobic interactions are most effectively realized in water and glycerol (as estimated by the values of critical concentrations of micelle formation); in the other solvents this ability is much lower.

These data allowed a criterion for the optimal solvent to be formulated (181). This criterion is universal, as the effectiveness of *solvophobic*, as well

as hydrophobic (185), *interactions is determined by the nature of the solvent rather than by the nature of the interacting molecules.* Consequently, if one assumes that the major role in the maintenance of the structure of protein molecules belongs to hydrophobic interactions (79), one should state that the solvents of the first class (and among them water used by nature and glycerol) are best for enzymatic reactions.

The validity of this criterion (181) is supported by data (172,180,186,187) that glycerol and to a lesser degree ethylene glycol really produce a lower denaturing effect on proteins than other solvents. That is why Klibanov et al. (181) succeeded in synthesizing in 95% wt/wt aqueous glycerol glycerophosphate from inorganic phosphate with a yield of 30% using acidic phosphatase as a catalyst. For the sake of comparison, in water the equilibrium in this reaction is strongly shifted toward the starting reagents (188).

Enzymatic Reactions in Biphasic Systems Such as "Water–Water-Immiscible Organic Solvent"

As immobilized enzyme research was developing, some works appeared describing how enzymes could be made to function in organic solvents (or aqueous–organic mixtures with a high concentration of organic component) by increasing their stability with the help of attaching them to a support (165,168,169,173,174,176,179,180,189–191). However, even in the best systems, at a concentration of a nonaqueous component exceeding 90%, immobilized enzymes do inactivate. This indicates that, from the point of view of preparative enzymatic synthesis, the problem is not resolved. Quite possibly, it defies solution at all, because when water is replaced by a nonaqueous medium the conformation of enzymes will inevitably change significantly (58,192), with their catalytic function being thereby impaired.

We found (59,182) a basically different solution to the problem. In all the works known to us where the behavior of enzymes in water–organic media was studied, a solvent such as acetone, acetonitrile, dioxane, dimethylsulfoxide, dimethylformamide, methanol, or ethanol was used as a nonaqueous component (see above). The common feature of all these solvents is their limitless ability to mix with water. This is why, in fact, the traditional problem arises of stabilization of enzymes against inactivation by an organic component. The gist of our approach (59,182) is that an organic solvent added to a water phase containing an enzyme should be *immiscible* with water, e.g., chloroform, ether, fatty aliphatic alcohols, or hydrocarbons.

In other words, the system should contain two phases, aqueous and organic. The enzyme will persist only in the aqueous phase, because common proteins possessing a satisfactory solubility in water are almost

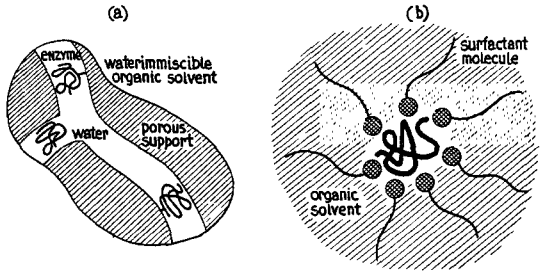
insoluble in hydrophobic solvents (58). If necessary, the enzyme can be immobilized in an aqueous phase (see below). The substrates, in their turn, when dissolved in the organic phase, can freely (193,194) diffuse to water, where they will undergo a chemical conversion, and the products will diffuse back to the organic phase. Thus the thermodynamic equilibrium in the chemical reaction will be established in the whole chemical reaction via water (with the help of the catalyzing enzyme dissolved in it).

Such a biphasic system possesses a number of basically important properties:

1. As the enzyme does not come into contact with the nonaqueous component of the reaction mixture, the problem of stabilization of the enzyme is eliminated (59,182).
2. The proportion of the organic phase volume can be made infinitely close to unity; hence the equilibrium in such biphasic system can be infinitely close to the equilibrium in the purely organic medium (see the theoretical notes given in Refs. 59,182).
3. A high content of the nonaqueous component in the medium can also ensure solubility of hydrophobic reagents (e.g., steroids) that is sufficient for preparative synthesis (195,196).

Methodological Aspects. The enzymatic system "water–water-immiscible organic solvent" may be prepared as an emulsion of an aqueous solution of the enzyme in an organic medium or, much more convenient methodologically and technologically, as a suspension in an organic medium of porous particles (e.g., porous glass or ceramics, hydrophilic gel) impregnated by an aqueous solution of the enzyme (59,182) (see Scheme 6a). The aqueous solution of the enzyme can be entrapped in microcracks of a polymeric carrier by its mechanical deformation (197). The enzyme can be used in both a free and an immobilized state.

It may seem that preparative enzymatic synthesis in a biphasic system cannot be applied to the reactions where the reagents (and, most of all,



SCHEME 6

reaction products) are ions, as they would not go from water to the organic phase. But this difficulty can be overcome (182) by selecting hydrophobic counterions for ionic reagents, as was the case in nucleotide chemistry (198) and as is the practice in phase-transfer catalysis (e.g., see Ref. 199).

Catalysis by Water-Soluble Enzymes Entrapped in "Reversed" Micelles of Surfactants in Organic Solvents

Molecules of many surfactants form in organic solvents associates of the "reversed micelle" type where polar (ionic) groups of the surfactant molecule make the nucleus of the associate and the hydrocarbon fragments constitute the external layer (200–202). Such surfactant micelles are known to be able to solubilize ions, polar organic substances, and also considerable amounts of water (several dozen water molecules per surfactant molecule). It has been demonstrated recently (183) that with the help of the micelle-forming surfactants, relatively high concentrations of biopolymers can be solubilized in organic solvents, amounting to 1 mg/ml, which corresponds to 10^{-5} M of active centers, with the molecular mass of the enzyme not exceeding 100,000; this by far exceeds the necessary level of the "catalytic concentrations" of the majority of enzymes (1,2). The enzyme almost completely retains its catalytic activity and substrate specificity. A molecule of the enzyme, when entrapped in a reversed micelle (see Scheme 6b), is protected against denaturation (unfolding of its structure) by the fact that the "interface" between the protein globule (or, respectively, the surface layer of water) and the organic solvent phase is stabilized by the surfactant molecules. As a result, the biocatalyst cannot come into direct contact with the unfavorable organic medium because it is in a "microreactor" containing only several hundred water molecules per molecule of the enzyme (which corresponds to less than 1% vol/vol of the total amount of water in the organic solvent surfactant system). For example, the relative catalytic activity of an enzyme (chymotrypsin was used for no special reason) solubilized in octane can be kept at room temperature for months (183).

Examples. All the above concepts have been verified (183) with chymotrypsin-catalyzed hydrolysis of nitroanilide of *N*-glutaryl-L-phenylalanine and peroxidase oxidation of ferrocyanide ions or pyrogallol. The reactions of these enzymes with their specific substrates were studied in rather hydrophobic (hydrocarbon) solvents, i.e., octane and benzene, with a surfactant commercially manufactured in thousands of tons, i.e., di(2-ethylhexyl) ester of sulfosuccinic acid sodium salt (Aerosol OT).

A typical experiment was the following: 2 ml of 0.1 M solution of the surfactant in hydrocarbon was supplemented with 0.01 ml (or less) of concentrated (10^{-3} M or less) solution of the enzyme in an aqueous buffer;

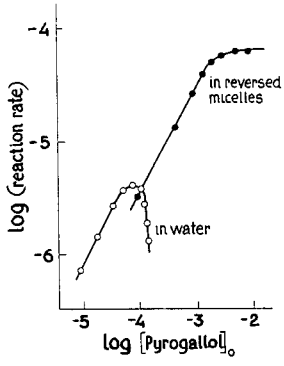


FIG. 11. Initial rate (M-min^{-1}) of peroxidase oxidation of pyrogallol (M) in water and in octane, with water content 2% wt/wt and 0.1 M Aerosol OT (micelle-forming component). Conditions: 26°C, pH 7, 2.5×10^{-3} M H_2O_2, 3×10^{-9} peroxidase. From Martinek et al. (183).

then 0.01 ml (or less) of the substrate solution in water or acetonitrile was added and the rate of the enzymatic reaction was measured spectrophotometrically in the resulting homogeneous (optically transparent) system.

Figure 11 presents the data on peroxidase oxidation of pyrrogallol. When water is replaced by a medium consisting of reversed micelles in octane, the kinetic mechanism of catalysis (as shown in Fig. 11) changes, because substrate inhibition, characteristic of reactions in water, is absent here.

Application to Fundamental Biochemical Research. Enzymes solubilized as suggested by Martinck et al. (183) in organic solvents (with addition of reversed micelle-forming surfactants) can be used as models for enzymes functioning in biological membranes in vivo and also to study the biological role of water in the structure of proteins and in the mechanism of enzymatic catalysis, especially in the processes where water acts as a chemical reagent. The amount of water solubilized in the system and consequently surrounding the enzyme molecule in a reversed surfactant model (see Scheme 6b) can be altered at will.

CONCLUSION

Stabilization of Enzymes Under the Action of Water-Soluble Additions

We have tried to describe the problem of enzyme stabilization in all its complexity, but have purposely left out the phenomenon (much used and widely discussed in the literature) of enzyme stabilization under the action of various effectors, such as bivalent metal ions (primarily Ca^{2+}, Mg^{2+}, Sr^{2+},

Zn^{2+}, Co^{2+}, and Mn^{2+}) (203–206), ammonium sulfate (207), various anions (primarily phosphate and sulfate) (208–211), serum albumin (212,213), polyols (primarily glycerol and also ethylene glycol, sucrose, etc.) (214–216), surfactants (217), and added organic solvents (218,219).

We have not discussed this kind of enzyme stabilization for the following reasons. First, the effects observed thereby are not of a general nature, as one effector can stabilize one enzyme and decrease the stability of the other (207,220), or one enzyme can be stabilized by one effector and labilized by another (221).

Second, most often the mechanism of enzyme stabilization by effectors is obscure, in other cases it is trivial; for example, the stabilizing effect of additions of serum albumin. This protein either effectively adsorbs on the interface (e.g., water/air), thereby preventing the inactivation of the enzyme itself, or sorbs cations of heavy metals and other admixtures and poisons the enzyme.

Third, this approach to the enzyme stabilization problem is not sufficiently technological, as the water-soluble stabilizing effector added can prove an undesirable admixture to the reaction product.

At the same time it should be emphasized that practical and theoretical work in this area is of great importance, especially if the research is methodologically correct and quantitative.

Two General Principles of Enzyme Stabilization

In the present communication, only sufficiently general physicochemical approaches that also have a certain technological value and allow the enzyme to be protected from the denaturing factors of the medium (elevated temperature, extreme values of pH, additions of organic solvents) have been considered. All of the various approaches discussed herein fall into two groups that correspond to the following two general principles:

1. Rigidification (fixation) of the protein globule, preventing it from unfolding due to denaturation and hence inactivation (Scheme 1). This can be achieved either by application of "brackets" on the protein (to form a water-soluble preparation) or as a result of a multipoint interaction (both covalent and physical) of the protein with the complementary surface of the support (to obtain an insoluble heterogeneous catalyst).
2. Spatial separation of the enzyme and the factors of the environment that are unfavorable for maintenance of the catalytic activity and specificity. Realization of this principle depends on the nature of the inactivating action. For example, to prevent pH inactivation, one

can use for immobilization of the enzyme a support either having buffering properties or capable of providing a local pH shift. The effect of the organic medium can also be coped with in two ways: either the enzymatic reaction is carried out in a biphasic "water–water-immiscible organic solvent" system or the enzyme is entrapped in reversed micelles of a surfactant.

Stabilization of Enzymes as a Problem Having an Optimal Solution

The methods for enzyme stabilization (against inactivation resulting from the unfolding of the protein globule) that have been so far elaborated can in some case produce negative side effects that will hinder technological usage of biocatalysis.

For example, when one is trying to stabilize the enzyme, one should not rigidify the protein globule too much; otherwise, the catalytic activity will be lost. The functioning of the active center presupposes that the action of several groups should be harmonious and hence requires mobility of the polypeptide chains (see references to Refs. 1,2). The interrelationship of the stability and the catalytic activity of the enzymes seems to be important in the adaptation of the organisms to alterations in environmental conditions such as temperature or the content of salts in marine water (54).

Furthermore, when an enzyme is entrapped in a support (e.g., in gel), the steric hindrances to the interaction of the enzyme with macromolecular substrates increase (see references to Refs. 5,65).

Finally, in supports that are too dense and ensure high thermostability of the immobilized enzyme, considerable diffusion hindrances can arise to the exchange of the reagents between the catalyst and the medium (see references to Refs. 5,65,66).

Consequently, in some cases, when one attempts to stabilize the enzyme, one has to solve a problem with an optimal solution, i.e., to optimize enzyme stabilization, so that having lost little in the catalytic activity the process gains in thermostability.

It is understandable that all these drawbacks are not inherent in the approaches based on spatial separation of the enzyme and the factors in the medium that are unfavorable for its functioning (see above).

Dependence of the Stabilization Effect on the Conditions in Which the Enzyme Functions

It should be remembered that the degree to which the enzyme can be stabilized depends not only on the conditions in which the enzyme was modified or immobilized but also (and to a great extent) on the conditions in

which the enzyme preparation obtained is to function. For example, Fig. 3 shows that above 50°C the enzyme entrapped in polymethacrylate gel is more stable than the free enzyme, whereas below this temperature the opposite situation occurs; the relative value of the stabilization effect changes in a similar way if one compares two preparations of immobilized enzymes. Moreover, in the latter case, the so-called isokinetic temperature (~82°C) could be recorded at which the two preparations have the same stability.

These data convincingly show how important it is to describe in great detail the conditions under which inactivation of an enzyme was studied, e.g., temperature, pH, buffer, ionic strength of the solution. Concentrations (both in the bulk and on the surface of the support) of the enzyme or/and ballast proteins were required for estimating the role of protein–protein interactions in inactivation (autolysis, association, etc.). While preparing for this review, we have analyzed a great many papers on the subject—and have found that these seemingly obvious requirements are not always met.

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