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The Stabilisation of Enzymes—a Key Factor in the Practical Application of Biocatalysis

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The present state of the problem of the practical application of biocatalysis is examined and physicochemical approaches whereby the denaturation of enzymes under the influence of elevated temperatures, extreme pH values, and organic solvents can be suppressed are analysed. The general principles of the stabilisation of enzymes are formulated.
The bibliography includes 225 references.

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I. INTRODUCTION

1. Practical Application of Biocatalysis

Enzymes as biocatalysts differ from the usual chemical catalysts by their exceptionally high catalytic activity under mild conditions and their unique substrate specificity[1,2]. This leads to wide prospects for their use as catalytic and regulating elements in various branches of science and engineering. A number of factors have interfered hitherto with the realisation of these possibilities: the low availability (and hence relatively high cost) of enzymes, high lability, and the difficulty of their separation from the reaction products.

However, in recent years the situation has altered radically. Firstly, the development of methods for the preparation of enzymes from micro-organisms has virtually solved completely the first problem[3-5], and one can claim confidently that, after a further improvement of microbiological technology, the cost of enzymes will decrease. Secondly, a new field, involving the preparation and study of immobilised enzymes[5-9], has been established at the junction between a number of chemical and biological disciplines[6]. As a result of immobilisation (for example the covalent binding of enzymes to water-insoluble particles, the inclusion of enzymes in a gel or hollow fibres, and the adsorption of enzymes on various carriers), the first benefit has been the possibility to separate the heterogeneous catalyst thus obtained from soluble

products and the ability to use it again. In many instances the stability of the enzymes has also been significantly increased. Because of this, the practical applications of enzymes have increased sharply. We shall indicate certain examples.

(1) The use of enzymes has been firmly included among the methods of analytical chemistry[9-11] and medicine[9,11].

(2) Particularly notable advances have been achieved in the fine organic synthesis of medicinal preparations and important biochemical compounds. Certain enzymic processes of this kind have already been carried out on the laboratory scale. As an example, one may mention the separation of the racemates of synthetic aminoacids[12,13], the synthesis of aminoacids[13-15] and antibiotics[15-17], the transformation of steroids[18,19], the synthesis of *N*-oxides of medicinal preparations[20,21], the synthesis of pyrrole porphobilinogen[9,22], the synthesis of hydroxynitriles[23], the preparation of pharmaceutically pure labelled aminoacids[24], the synthesis of important biochemical substances such as ATP,[25] NAD,[26] and others[27], and the synthesis of homopolynucleotides[28,29]. There is no doubt that the application of enzymes in preparative organic synthesis will develop vigorously also in the future[9,30,31].

(3) The use of enzymes has served as a powerful stimulus in the development of new large-scale technologies, such as the preparation of glucose by the hydrolysis of starch[32] and the isomerisation of glucose to fructose[33], the synthesis of L-DOPA and L-methionine[34], and reduction of the lactose content in milk[35,36]. Research into the possibilities of the industrial preparation of glucose from cellulose with the aid of cellulase is being vigorously prosecuted[37].

(4) Approaches to the preparation of enzymic light-sensitive[38] and sound-sensitive[39] materials, which can serve as chemical amplifiers of weak signals (particularly for the creation of new photographic and sonographic processes), have been developed.

(5) Methods for the solution of energy problems of the future with the aid of enzymes have been outlined[5,7,40].

The above list of problems which have been already solved or merely formulated cannot be regarded as complete and it can be extended (see, for example, Refs.5–9 and 41); however, we shall concentrate attention on the discussion of the causes which for fundamental reasons still limit the application of enzymes as catalysts of chemical reactions of practical importance, namely the inactivation of enzymes under technological conditions.

2. The Causes of the Inactivation of Enzymes

The loss of observed catalytic activity by the system as a whole can be due, for example, to the elution of the immobilised enzyme from within the carrier or from its surface or the partial breakdown (or deformation) of the carrier itself by both mechanical and microbiological means[42,43]. These and similar technological aspects[43] are of only partial interest, because the observed inactivation of this kind can be readily prevented by choosing another carrier or by altering the method for the immobilisation of the enzyme.

The following inactivation processes, in which the enzyme itself (its active centre) is "destroyed" are more important.

A. The enzyme can be "poisoned" by inhibitors such as heavy metal cations: in order to avoid this, one can add a metal-chelating agent of the type of EDTA [44] either in a free form (this appears more promising) or in an immobilised form[45].

B. The catalytic activity can decrease as a result of the oxidation of the functional groups of the protein. In this case protective agents must be introduced into the system. In principle, one can employ for this purpose antioxidants [(inhibitors of free radical reactions, which have already proved useful in the solution of other applied problems in biochemistry and medicine (see, for example Emanuel'[46])]. Sulphydryl compounds are used to protect the SH groups[47]. The oxidative degradation of enzymes can be eliminated by employing anaerobic conditions (in principle, a very low oxygen concentration can be maintained in the system by absorbing oxygen via auxiliary autocatalytic oxidative processes or even with the aid of enzymic oxidative processes coupled with the main reaction.

C. The "eating" of the protein catalyst by micro-organisms (they are almost always present in the system as an impurity) can be avoided by immobilisation if the enzyme is incorporated in a carrier (for example a microporous carrier) which protects it as a result of steric hindrance[5,42,43].

D. Covalent immobilisation also prevents various kinds of polymolecular inactivating processes of the type of enzyme–enzyme interaction, such as aggregation[48] or autolysis in the case of proteolytic enzymes[5,7].

E. When used in flow reactors, the enzyme can be inactivated as a result of the elimination of the cofactor from the system. However, recently this problem has been solved in principle. There exist three most promising (from our point of view) approaches: (a) a membrane reactor is used and the cofactor is attached to a water soluble polymer[49]; (b) the enzyme (enzymes) and the cofactor are jointly subjected to covalent immobilisation[49]; (c) more complex enzyme systems are created in which the cofactor is regenerated[25,49,50].

F. Inactivation of enzymes caused by the denaturing activity of the external medium. Since methods for the suppression of inactivation via mechanisms (A)–(E), developed to a greater or smaller extent, already exist, in the present review we shall deal only with mechanism (F). There is naturally no special point in considering the problems of preventing the denaturation of enzymes under the influence of the carrier[51]—it is simplest to use another carrier. We shall have in mind primarily the following denaturing factors: increased temperature, unfavourable pH values, and the addition of organic solvents.

3. The Necessity to "Suppress" the Denaturation of Enzymes

The prevention of the denaturation of enzymes is an essential step towards their wide-scale practical application. We shall demonstrate this on the following examples.

1. When enzymes are extracted from their natural environment (*in vivo*), they become as a rule labile and in many instances their "lifetime" is measured in minutes[52-54]. For example, the stability of immobilised aspartase proved to be unsatisfactory for its use in industry[55].

2. It is desirable to carry out many processes at an elevated temperature. This is associated, in the first place, with the fact that the rates of the majority of chemical reactions, including enzymic reactions, increase with increasing temperature (in accordance with the Arrhenius equation). It is therefore natural that at elevated temperatures the specified degree of conversion of the substrate in the chemical reactor is attained after a shorter period or even in the presence of smaller amounts of the immobilised enzyme, and in many industrial processes the cost of the enzyme is an important economic factor, for example, in the case of glucoamylase[32]. In the second place, an increase of temperature ensures bactericidal conditions[43], which are very important, for example, for processes in the food industry[35, 56,57]. However, we may note that at elevated temperatures the denaturation of biocatalysts intensifies[52-54].

3. The equilibrium position in many processes of practical importance is such that the required products can be obtained when the reaction is carried out in a mixture of water and an organic solvent with a high content of the latter. On the other hand, enzymes as a rule lose their catalytic activity or specificity under these conditions[53,58] (see also the references in the paper of Klibanov et al.[59]).

4. In certain cases the pH optimum of the reaction and the pH range corresponding to the stability of the enzyme catalysing it are not the same. A typical example is in the synthesis of penicillin antiobiotics under the influence of penicillin amidase: the equilibrium position in the synthesis is displaced towards products in an acid medium, where the enzyme is fairly unstable[60].

5. Evidently in the practical employment of enzyme preparations their direct application takes place long (sometimes very long) after their preparation. This gives rise to the problem of the stabilisation of enzymes in relation to their inactivation on prolonged storage[61].

4. Methods for the Solution of the Problem of Undesirable Inactivation

In order to solve the problem of the undesirable inactivation of enzymes, a number of approaches have been proposed. Firstly, one can use whole (or only partly degraded) cells as the catalyst without extracting the enzyme from its natural environment[4-9]. For example, owing to the low stability of free aspartase, immobilised cells of *Escherichia coli* had to be used for the continuous synthesis of L-aspartic acid[55]. Partly degraded cells retain their catalytic properties even in organic solvents[62]. Secondly, the enzyme can be isolated from specially stable, for example, thermophilic, strains[54,63].

However both these approaches have their disadvantages. The use of whole cells creates the problem of the diffusion of the substrates or products through cell walls; furthermore, the choice of the method of immobilisation is in this case evidently limited. The use of enzymes from thermophilic microorganisms is limited by the fact that the search for strains suitable in the required catalytic reaction constitutes in each case a special (individual) problem, which is by no means simple.

In the present review, we shall therefore consider the third possible solution—the stabilisation of an enzyme molecule (globule) in relation to denaturing influences by artificial means.

The stabilisation of enzymes could not be achieved "at a stroke" by their immobilisation: the stability of enzymes in relation to inactivating influences in some cases increases on immobilisation, in others it decreases, while in instances of a third kind, it remains virtually unaltered[6]. Certain advances in the formation of enzyme preparations with an increased stability have therefore come to be regarded[7,42] as an exception rather than a rule. Furthermore a large text published in 1976 and devoted to immobilised enzymes[9] altogether failed to provide a genuine treatment of this problem.

II. THE STABILITY AND STABILISATION OF ENZYMES; DEFINITIONS AND GENERAL REMARKS

The stability of an enzyme *E* will be understood as its ability to retain its intrinsic catalytic activity under conditions where the inactivating mechanisms (A)–(E) are absent. The effective first-order rate constant, characterising the unimolecular inactivating process

$$k_{inact} = (d[E]/dt)/[E],$$

where [E] is the relative specific catalytic activity, will serve as a quantitative criterion of the stability of the given enzyme under the given conditions.

The decrease of k_{inact}, observed as a result of a particular treatment of the enzyme (modification, immobilisation, etc.), will serve as a measure of stabilisation.

In certain systems, an apparent stabilisation of the immobilised enzyme in relation to its native precursor is observed, being caused, for example, by diffusion-dependent obstacles to the enzymic reaction, which arise under the influence of the carrier[64]. We shall consider only studies where complications of this kind have been excluded; we shall make use of the kinetic criteria formulated by Berezin et al.[65] (see also Goldstein[66]).

The problem of the stabilisation of enzymes in relation to denaturing influences does not have only practical aspects of the kind discussed above. It is important also for the study of the mechanisms of the denaturation of enzymes. Furthermore, certain stabilised enzyme systems serve as models of systems functioning *in vivo*. All these aspects will be considered in the present review.

Below we shall analyse methods (mechanisms) of stabilisation, which, being to some extent general, in the first place do not reduce unduly the initial level of the catalytic activity of enzymes extracted from their natural environment (as a rule, not more than by a factor of 2–3) and, in the second place, ensure the continuing functioning of the enzyme under unfavourable conditions in the surrounding medium. We shall consider separately the preparation of water-insoluble (truly immobilised) heterogeneous catalysts with an increased stability and, on the other hand, stabilised water-soluble enzyme preparations. The need for soluble enzymes has been continuously increasing in recent years[5,67], primarily in medicine[5,9,11,68] and in chemical engineering, in processes involving the use of water-insoluble substrates[69]; the cellulase cleavage of cellulose to glucose is particularly promising in this connection[37].

However, we shall in no way endeavour to compile fully all the studies in which the enzyme stabilisation effect has been observed (a continuously increasing number of such studies apparently precludes this within the framework of a single article). We shall concentrate attention only on those investigations where the observed effects have been subjected to an experimentally tested physicochemical treatment; the number of such studies is relatively small. The main aim of this review is to formulate (and to illustrate by the most striking examples) the physicochemical principles of the solution of the problem of the stabilisation of enzymes. We shall begin with the fact that enzymes are inactivated via identical mechanisms (see above) under both technological conditions and on storage. We may note that the stability of enzymes is usually considered separately in the two cases; this is justified in view of the fact that, under particular conditions, the contribution of each inactivating mechanism can be signifcantly different. For example, at an elevated temperature (in technological processes) mechanisms involving the denaturation uncoiling of the protein or its oxidation largely operate, whereas during storage (usually at a reduced temperatue) one may expect mainly the degradation of the enzymes under the influence of micro-organisms and proteases (particularly in a moist state). For attempts to stabilise the enzyme to be well-founded, it was therefore necessary to begin in the first place with the causes of the inactivation; it is even better (although not essential) to know the mechanism of the inactivation.

III. THE STABILISATION OF ENZYMES IN RELATION TO THERMAL INACTIVATION (IN RELATION TO THE UNCOILING OF THE PROTEIN GLOBULE)

As a result of the modification of enzymes by chemical reagents (including water-soluble synthetic reagents or biopolymers) or their attachment to a water-soluble carrier, their thermal stability is frequently altered (both decreased and increased). This is usually explained (see, for example Refs.5–7, 42, and 70) by the following causes: (1) the change in the conformation of the enzyme molecule compared with its native structure on the assumption that the thermal stability of the protein globule depends on its conformational state; (2) the change in the microenvironment of the enzyme molecule, assuming that it influences the intramolecular links (factors) which maintain its native structure.

These views, however valid they may be, cannot serve as a basis for the development of general methods for the stabilisation of enzymes. The point is that we do not know in what way the stability of the enzyme is related to its conformation and microenvironment; furthermore, even if this relation actually exists†, it ought to be, in all probability, specific to each enzyme.

The fact that the overall fundamental mechanisms of the inactivation of enzymes still remain unelucidated[52] although vigorous research in this field has continued for many decades also hinders the development of rational ways of increasing enzyme stability. It is extremely likely that a single general mechanism of the denaturation of proteins does not exist al all[77]. However, there is no doubt that the inactivation of enzymes under the influence of, for example, heat or denaturing agents is accompanied by considerable conformational changes in protein molecules, i.e. their uncoiling[52–54,77]. It therefore appears evident that an increase of the thermal stability of enzymes can be achieved by yet another procedure, namely if (3) the native conformation of the protein globule is made more rigid (is fixed).

In fact, if it is recognised that a necessary stage in the inactivation of the enzyme is the uncoiling of its molecule, then, the more rigidly (firmly) is the protein globule of the enzyme fixed, the greatly the difficulty of uncoiling it and hence of inactivating its active centre. One should note that this approach (involving a variation of the rigidity of proteins) is widely used in nature in the adaptation of organisms to the conditions in the environment[54].

The fixation of the enzyme globule to prevent its uncoiling can be achieved in several ways: by the introduction of cross links (1), by covalent (2) or non-covalent (3) attachment to a carrier, and by mechanical inclusion (4) in the narrow pores of a carrier. We shall consider each of these approaches in greater detail.

1. Intramolecular Cross Links and Their Influence on the Thermal Stability of Enzymes

There is extensive information in the literature about methods for the chemical modification of enzymes and the properties of the resulting preparations. However, one can hardly predict in a single instance how the thermal stability of the enzyme will alter as a result of its chemical modification. This is associated with the exceptional complexity of the molecular structure of biocatalysts (which, in addition, are still unknown for many enzymes). As an example of the extremely complex dependence of the thermal stability of an enzyme on the degree of modification of its surface layer, we shall indicate the behaviour of α-chymotrypsin alkylated via its NH_2 groups[105]. The enzyme was modified by treating it with acroleine with subsequent reduction of the Schiff bases by sodium tetrahydroborate (using the methods indicated by Feeney et al.[106]); this entailed hardly any loss of catalytic activity by the enzyme. Fig.1 shows that the alkylation of the NH_2 groups, which are the first to be modified, also hardly affects the thermal stability of α-chymotrypsin. The modification of the least reactive NH_2 groups (apparently relatively inaccessible groups deep in the globule) plays a decisive role: the thermal stability of the enzyme increases sharply in the concluding state of its modification (by two orders of magnitude); on further alkylation, the thermal stability of the enzyme preparation falls (as a result of the complete modification of all the NH_2 groups titratable by trinitrobenzenesulphonic acid). Fig.1 shows that the observed effect (a sharp increase of thermal stability with a subsequent sharp fall) is manifested to the maximum extent when only two-thirds of the NH_2 groups, the total number of which is 17, are modified.

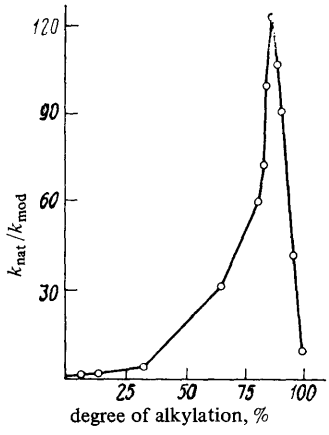


Figure 1. Dependence of the pseudo-first-order rate constant (k_{mod}) for the unimolecular thermal inactivation of α-chymotrypsin on the degree (based on titration data) of alkylation of the NH_2 groups by acroleine (with reduction of the Schiff bases by sodium tetrahydroborate); the value of k_{nat} obtained for the native (unmodified) enzyme was adopted by convention as unity. Conditions: 50 °C, pH 8 (5×10^{-3} M tris.HCl + 0.1 M KCl).[105]

The situation is different when bifunctional reagents, capable of cross-linking covalently the protein globule, are used: here one may expect an increase of the conformational stability of the protein molecule[78]. This approach therefore leads to wide prospects for the preparation of water-soluble stabilised enzyme preparations, which may find practical applications (see Section I).

Intramolecular cross links in nature. Nature makes an extensive use of intramolecular cross links for increasing the rigidity (and hence the stabilisation) of protein molecules[54].

† Thus it has been shown in recent studies by direct physical methods that the attachment of proteins to carriers does not lead to any significant conformational changes in their macromolecules[71–76].

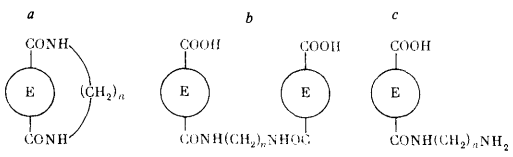
These cross links can be both covalent bonds (disulphide bonds[79-81]) and weaker linkages ("salt bridges"[82], or, for example, Ca^{2+} ions incorporated into the protein molecules[83], etc.).

The artificial introduction of cross links ("clamps") into protein molecules can be achieved by treating them with bifunctional reagents[11,84,85]. Dialdehydes, diimidoesters, diisocyanates, bisdiazonium salts, etc. are usually employed as reagents of this kind. Analysis of literature data[67,78,86-94] shows that in most cases the stabilisation effects are extremely small and sometimes are apparently in fact caused not by the cross links but by simple (single-point) chemical modification of the enzyme[67] as shown recently for glutaric dialdehyde[92]. In other cases the stabilisation effect proved to be more appreciable[78,91], an increase of stability being correlated with the increase of the rigidity of the protein molecule[78].

In all probability the success or failure of the attempt to increase the stability of the enzyme by treating it with bifunctional reagents, depends mainly on the extent to which the length of the bifunctional reagent corresponds to the distance between the possible centres of cross links in the protein globule. Evidently, for each given protein there is an optimum size of the intramolecular cross-linking agent. Studies directed to the selection of the optimum size of the bifunctional reagent are therefore of greatest interest.

Variation of the length of the intramolecular cross link. Studies of this kind are rarely performed, probably because the bifunctional reagents used directly to introduce cross links are commercially unavailable and their chemical synthesis is extremely laborious. It is therefore a very attractive propostion to employ bifunctional reagents which cannot be used directly to cross-link the enzymes intramolecularly but become suitable if the enzyme is subjected to an appropriate preliminary modification. A number of aliphatic diamines, which are commercially available, have a carbon chain comprising between 0 and 12 methylene groups, and are fairly cheap, proved useful for this purpose[95]; both factors are particularly important for possible practical applications. The diamines can be used to cross-link the carboxy-groups of enzymes (which are usually fairly numerous in proteins) provided that they are first activated with carbodiimide. This results in the formation of a strong amide linkage between the amino-group of the modifying agent (the diamine) and the carboxy-group of the protein[96]:

Scheme 1



Under conditions where there are no intermolecular cross links between the protein molecules (leading to the formation of enzyme oligomers—see Scheme 1b), it has been found that[95] there is a significant increase of the thermal stability of α-chymotrypsin intramolecularly cross-linked by this method (relative to the native preparation, the thermal inactivation rate constant decreases by a factor greater than 40). The observed stabilisation effect cannot be explained by simple (single-point) chemical modification of the enzyme (Scheme 1c), because the modification of chymotrypsin by a monoamine (provided the enzyme is subjected to preliminary activation with carbodiimide), namely 1-amino-3-propanol, actually reduces its thermal stability. The stabilisation effect depends on the number of methylene groups n in the molecule of the modifying agent $H_2N-(CH_2)_n-NH_2$. Fig.2*a* shows that the dependence of the first-order reaction rate constant (observed for the thermal inactivation of chymotrypsins "cross-linked" by various diamines) has a minimum at $n = 4$, although the stabilisation of the enzyme is also observed for $n = 2$, 5, and 6.

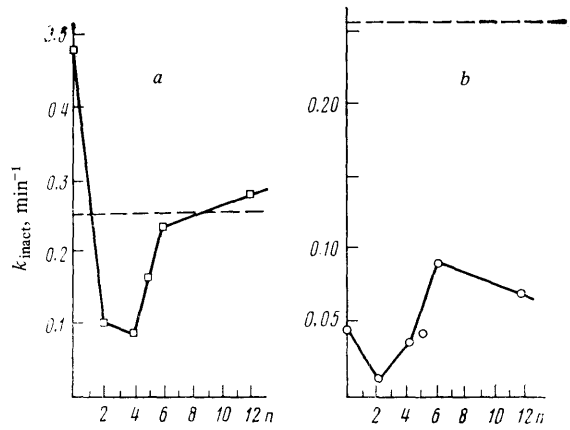


Figure 2. Dependence of the first-order rate constant (k_{inact}) for the unimolecular thermal inactivation of α-chymotrypsin (*a*) and succinylated α-chymotrypsin (*b*) cross-linked intramolecularly by diamines on the number of methylene groups in the molecule of the modifying agent $NH_2(CH_2)_nNH_2$.[95] The dashed line represents the level of the thermal stability of the native and succinylated enzymes. Conditions: pH 7 (0.02 M phosphate), 50 °C.

The fact that treatment with tetramethylenediamine leads to the maximum stabilisation of chymotrypsin is apparently due to the optimum correspondence between the length of the above cross-linking agent and the distance between the carboxy-groups of the protein molecule; this leads to the formation of a greater number of intramolecular cross links than for other diamines.

The grafting onto the enzyme of ability to interact with bifunctional (polyfunctional) reagents. Presumably the greater the number of clamps (cross links) applied to the protein molecule, the greater its stability in relation to uncoiling and hence inactivation. The possible number of cross links is determined in its turn by the number and relative positions of functional groups on the surfaces of the protein interacting with the cross-linking agent. Clearly their number (and hence the number of possible cross links) can be increased by the appropriate modification of the protein.

Reiner et al.[97] suggested that SH groups be introduced into the enzyme molecule for this purpose (by means of *N*-acetylhomocysteine thiolactone or other compounds)[98-100] with subsequent employment of the bisiodoacetamides derived from various aliphatic diamines as bifunctional reagents. For the system described above (Fig.2*a*), it proved useful to subject the enzyme to a preliminary modification with succinic anhydride in order to increase the content of carboxy-groups in its surface layer[101]. The succinylated chymotrypsin was then treated (by the same method as the native

enzyme) with diamines of different lengths. Fig.2*b* illustrates the relation obtained under these conditions[95]. Evidently, firstly the maximum enzyme stabilisation effect has increased; secondly the maximum stabilising influence is shown by the shorter bifunctional reagent ethylenediamine and not tetramethylenediamine (cf. Fig.2*a*). The latter finding indicates a denser "population" of the surface of the succinylchymotrypsin globule (containing native chymotrypsin) by carboxy-groups.

The use of intramolecular cross links in fundamental biochemical research. Studies designed to select the optimum size of the cross-linking agent are not only important from the practical point of view (in order to obtain stabilised enzyme preparations) but can also yield new information about the tertiary structure (conformation) of proteins in solution[84] (for example, the distance between the functional groups in the surface layer of the protein globule can be estimated[102]), about the topography of the active centre (see the reference in the paper of Kennedy[85]), about the symmetry of the distribution of subunits in oligomeric proteins[85,102], about cooperative and allosteric effects[104], etc.

2. Multipoint Covalent Attachment of the Enzyme to the Complementary Surface of the Carrier

The methods for the covalent attachment of enzyme molecules to different carriers have been described fairly completely in numerous reviews (see, for example, Refs.5–9 and 11). This immobilisation procedure can promote, in principle, an increase of the conformational stability of the protein molecule and hence can prevent its uncoiling and inactivation. Thus the study of the conformational mobility of immobilised proteins (using various physical methods) showed that the attachment of the protein to the carrier makes the molecular structure of the former more rigid (it becomes "frozen")[71,72,74]. The same conclusion can be arrived at on the basis of many indirect data (obtained by both physical methods and by measuring the enzyme activity)[72,74,107–109]: after such proteins have been grafted to the carrier, the conformation transitions in them are greatly hindered. At the same time, Gabel showed in his pioneer investigation[110] that the greater the number of linkages attaching trypsin to Sephadex, the greater its resistance to inactivation by urea (under the influence of which the protein is known to become uncoiled[111]).

Thus the above findings suggest that the general principle governing the stabilisation of enzymes involves *multipoint* attachment of the catalyst molecule to the carrier. Such grafting should make the conformation of the catalyst molecule more rigid (incidentally it may not be otherwise altered in this process) and hence more resistant to uncoiling and inactivation. However, this principle is extremely difficult to apply methodologically because of steric factors: both the surface of the carrier and the surface of the protein have their individual reliefs which, generally speaking, are not complementary. Furthermore, it is evident that even in the case of multipoint interaction, when the enzyme is connected to the carrier solely by a small section of the surface, it is difficult to expect a significant increase of the rigidity of the entire molecule. Presumably this is why many attempts to stabilise enzymes failed[71,112,113]. The problem in fact reduces to creating a carrier surface which is rigorously complementary to the surface of the enzyme molecules. Only then is it possible to create a multipoint interaction between the protein and the carrier favourable for stabilisation.

The creation of a polymeric carrier complementary to the molecule of the immobilised enzyme. The essential feature of the approach permitting a solution of this problem[114–116] is as follows. The enzyme is modified by an analogue of the monomer and the resulting preparation is then copolymerised with the monomer itself (and in certain cases also with the cross-linking agent). Ultimately one obtains an enzyme which is chemically built into the three-dimensional network of the polymeric gel, the centres of the preliminary modification of the enzyme molecule serving as the points of attachment of the enzyme to the carrier. Evidently, by virtue of the very principle of the proposed method, the microsurface of the gel near the protein molecule built into it will be complementary to the surface of the protein.

A method for the preparation of enzymes immobilised by copolymerisation has been developed by Jaworek et al.[117], who endeavoured to prevent the elution of immobilised enzymes from gel carriers. The authors of the present review[114–116] developed this method independently in order to stabilise enzymes. It is very convenient to employ as monomer analogues acylating and alkylating agents containing a double bond capable of copolymerisation, for example, acryloyl chloride (which acylates the NH_2 and OH groups of the protein) or acroleine[114] (which interacts with the NH_2 groups; when the Schiff bases formed are reduced with sodium tetrahydroborate, the protein is found to be alkylated); a method has also been developed for the vinylation of the carboxyl-groups of the protein[118]. Acrylamide, sodium methacrylate, and ethylene methacrylate, which are frequently used to synthesise gels, can be used as the monomers and the cross-linking agent can be *N,N'*-methylenebisacrylamide. All these reagents are commercially available and cheap.

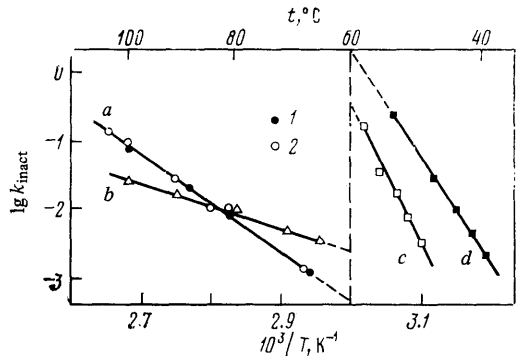


Figure 3. Temperature variation of the pseudo-first-order rate constant (k_{inact}, min^{-1}) for the unimolecular thermal inactivation of acryloylchymotrypsin incorporated in polymethacrylate (*a*) and polyacrylamide (*b*) gels [1) water-insoluble enzyme; 2) water-soluble enzyme], native α-chymotrypsin (*c*) and α-chymotrypsin modified by acryloyl chloride (*d*). Conditions: pH 8 (0.005 M tris.HCl+3 M KCl).[114]

The magnitude of the stabilisation effect. Fig.3 presents, as a striking example, data, expressed in terms of the Arrhenius coordinates, for the rate of thermal inactivation of α-chymotrypsin built into polymethacrylate and polyacrylamide gels (straight lines *a* and *b*)[114]. The resistance of the

immobilised enzyme preparations to heating is so much greater than that of the native enzyme (line *c*) that a direct, i.e. at the same temperature, experimental comparison is impossible. It is therefore necessary to compare the thermal inactivation rate constants determined by extrapolation, for example, at 60 °C (the vertical dashed line in Fig.3). At 60 °C acryloylchymotrypsin built into polymethacrylate (line *a*) and polyacrylamide (line *b*) gels is respectively 1000 and 200 times more stable than the native enzyme (line *c*). Compared with free acryloylchymotrypsin (line *d*), an even more striking difference is observed.

Fig.3 shows that the activation energies for the thermal inactivation of acryloylchymotrypsin built into both polymethacrylate and polyacrylamide gels are much lower (35 and 15 kcal mol^{-1} respectively) than for the native enzyme (110 kcal mol^{-1}). From this it follows that, with increase of temperature, the degree of stabilisation of the immobilised chymotrypsin will increase compared with the native enzyme. Thus extrapolation of straight line *c* in Fig.3 shows that at 102 °C the enzyme built into the gels is more stable than the native enzyme by a factor greater than 10^8.

Dependence of the stabilisation effect on the number of links between the immobilised enzyme and the carrier. It is noteworthy that the stabilisation effect in systems discussed above (Fig.3) is independent of both the initial density of the gel carrier (i.e. of the polymer concentration or more correctly of the monomer concentration) in the polymerisation mixture in the range from 10–15 to 50 wt.% and of the degree of its swelling (which depends on the amount of cross-linking agent, namely *N,N'*-methylenebisacrylamide). Furthermore, if the acrylic derivatives of the enzyme are copolymerised with the comonomers altogether in the absence of a cross-linking agent, the resulting gel-like polymer can be fully dissolved in water and, what is most important, the enzyme preparation obtained has the same high thermal stability as the enzyme immobilised in an insoluble gel (Fig.3*a*)[114]. This phenomenon is apparently due to the fact that the polymeric structure of the gel in the vicinity of the enzyme molecule, being complementary to its surface, is specified by the number of cross links with the protein.

The degree of covalent binding of the copolymerised enzyme to the carrier exerts in its turn a decisive influence on the thermal stability of the enzyme, namely, with increase of the number of cross links between the immobilised enzyme and the carrier, there is an increase in the thermal stability of the catalytic activity in the system. Thus Fig.4 shows that the effective rate constant for the thermal inactivation of α-chymotrypsin (at 60 °C) falls by a factor greater than 1000 when the degree of binding of the enzyme to the polymethacrylate carrier increases (the number of residual free NH_2 groups after acryloylation was determined experimentally by titration)[114].

The stabilisation mechanism. The observed enzyme stabilisation effect cannot be explained by the possible change in the thermal stability of the enzyme as a result of chemical modification, because free acryloylchymotrypsin is thermally even less stable than the native enzyme (Fig.3). Nor can stabilisation be explained by the influence of the micromedium in the gel on the enzyme, because the thermal stability of chymotrypsin physically incorporated in the polymethacrylate gel at the same concentration is virtually the same as that of the free enzyme[119,120] (Fig.5). The nature of the gel is also not a decisive factor for the stabilisation of the enzyme—analogous effects have been obtained for both polyelectrolyte (polymethacrylate) and electrostatically neutral (polyacrylamide) gels (Fig.3). The stabilisation effect occurs when different methods are used for the preliminary modification of the enzyme: alkylation of the NH_2 groups by acroleine or acylation of the protein by acryloyl chloride (Fig.3)[114] or vinylation of the carboxy-groups[118]. Finally, stabilisation has been achieved for different enzymes: α-chymotrypsin, trypsin[114], and penicillin amidase[121]. Consequently the very fact that the enzyme molecule is built into the three-dimensional network of the gel and not the method of preliminary modification or the nature of the gel is of decisive importance. Evidently the stabilisation mechanism depends on the fact that the uncoiling of the globule on heating is difficult in a fixed state of the protein. Here one must emphasise that the native (catalytically active) conformation of the active centre can be preserved only if the enzyme is bound to the carrier via numerous points of attachment (Fig.4).

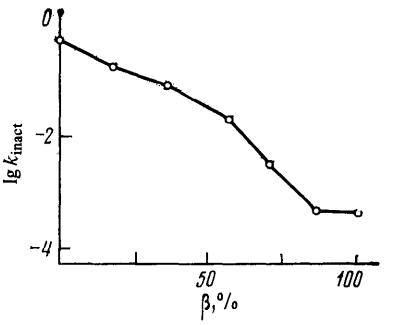
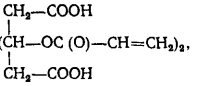


Figure 4. Dependence of the pseudo-first-order rate constant (k_{inact}) characterising the unimolecular thermal inactivation of α-chymotrypsin incorporated in a polymethacrylate gel on β (the degree of covalent binding of the enzyme to the carrier). Conditions: pH 8 (0.005 M tris.HCl + 3 M KCl), 60 °C.[114]

Further prospects. There is no doubt that the thermal stability of the copolymerised enzyme must depend (for the specified number of cross links between the protein and the carrier) on the rigidity (flexibility) of the polymer chains linked covalently to the protein globule and maintaining the native structure of the latter: for more rigid (less flexible) polymers (we have in mind this property at the molecular level), one should expect a greater stabilisation effect[115]. This principle must be tested experimentally in the future. Protein modifying agents and comonomers containing in their molecule at least two polymerisation centres and hence ensuring the formation of branched (completely cross-linked) polymeric gel structure may prove to be useful for this purpose. In order to ensure an adequate solubility, these compounds must be electrolytes, for example, the compound



which must be synthesised by acylating tartaric acid by acryloyl chloride. Furthermore, the polyelectrolyte nature of the resulting polymer might ensure an additional increase of the rigidity of its chains[122].

Gels suffer from a number of disadvantages as carriers[123], which include, in particular, low mechanical strength and a tendency of the gel grains to deteriorate on storage. However, these disadvantages can be avoided if the polymerisation is carried out within the pores of fairly stable carriers, for example, inorganic carriers, which have proved to be very useful[32,124,125]. The covalent attachment of the enzyme to elastic carriers (including gel-like carriers) leads to the possibility of regulating the catalytic activity by mechanical means[126]. In the case of polyelectrolyte carriers the catalytic activity of the immobilised enzyme can also be regulated by altering the ionic strength of the solution[127]. These aspects of the regulation can prove to be useful when enzymes are employed in organic synthesis (in chemical engineering).

The use of covalently immobilised enzymes in fundamental research has been very fully described by Mosbach[9].

3. The Multipoint Non-Covalent Interaction between the Enzyme and the Carrier

The multipoint interaction between the enzyme and the carrier can ensure, in principle, the stabilisation of the biocatalyst even when physical immobilisation methods are used. Physical immobilisation methods (for example, mechanical inclusion in gels[123], microencapsulation[128], adsorption on carriers[129,130], etc.) have been developed in fair detail[5,9,11], but the thermodynamic causes (mechanism) of the thermal stabilisation of enzymes in such systems have been discussed only recently and recommendations have been made for the formation of stabilised enzyme preparations[119,120]. As an example, we shall consider in greater detail the thermal stability of α-chymotrypsin incorporated mechanically in a polymethacrylate gel. In principle, the enzyme can form weak electrostatic or hydrogen bonds with this polyelectrolyte matrix.

The magnitude of the stabilisation effect. Fig.5 presents the variation of the logarithm of the effective rate constant for the thermal inactivation of α-chymotrypsin with the concentration of the polymethacrylate gel (up to the limit of the solubility of the monomer in water). Evidently, when the gel concentration is varied from 0 to 30 wt.%, the rate of thermal inactivation (at 60 °C) of the enzyme is almost constant, but, on further increase of the gel concentration, it falls very rapidly: in 50% gel the stabilisation effect reaches 10^5. Furthermore, extrapolation of the linear relation between $\lg k_{inact}$ against $1/T$ showed[119] that the lifetime of the enzyme immobilised in a polymethacrylate (45 wt.%) gel can amount to millions of years at room temperature.

The stabilisation mechanism depends on the interaction between the enzyme molecules and the polymer matrix. In principle, protein molecules can form electrostatic and hydrogen bonds with the carboxy-groups of the carrier, i.e. with polymethacrylic acid. However, these bonds are relatively weak; this is probably why they are hardly formed in a polymer solution or in dilute gels, where the formation of such a complex would entail considerable entropy losses in the "quenching" of the translational as well as rotational movements of the enzyme molecules.

The situation should be different in concentrated gels, where one may expect that the translational (possibly rotational) movement of protein molecules is significantly retarded (regardless of the formation of the complex) by virtue of steric hindrance introduced by the dense three-dimensional polymer network. Consequently in a concentrated gel the formation of a protein–matrix complex should be thermodynamically much more favourable, because there is no expenditure of free energy (or it is significantly reduced) caused by the loss of translational and rotational entropy by the enzyme globule when it is sorbed on the polymer matrix. One cannot rule out the possibility that in a concentrated gel the mobility of the polymer chains themselves is somewhat limited, so that the entropic losses of free energy, necessary for the local "freezing" of the matrix on formation of its complex with the enzyme, are therefore also significantly reduced compared with dilute gels (or a polymer solution).

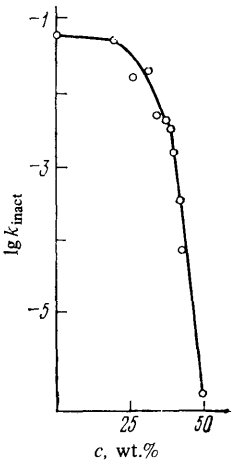


Figure 5. Dependence of the pseudo-first-order rate constant (k_{inact}, s^{-1}), characterising the unimolecular thermal inactivation of α-chymotrypsin incorporated mechanically in a polymethacrylate gel, on the concentration c of the polymethacrylate in the gel. Conditions: pH 8, 60 °C.[119]

Finally, under the conditions of a concentrated gel one may expect that the interaction of the enzyme with the matrix should be of the multipoint type for purely steric reasons, because polymer chains will approach the enzyme globule from all sides. Such multipoint interaction can lead to a sharp increase of the thermal stability of the immobilised enzyme (as already shown above for the covalent enzyme–carrier interaction).

All these postulates have been proved[119] as a result of a study of the thermal stability and the translational and rotational mobilities of enzyme molecules as a function of the gel concentration.

The proposed mechanism[120] operates also in other systems investigated previously. The specific protein–carrier interaction can also be of a type other than in the case discussed above, where electrostatic and hydrogen bonds are formed. We shall consider data[131,132] for the thermal stability of acetylcholinesterase, glucose oxidase, and peroxidase incorporated mechanically in the hydrophobic organosilicon gel silastic. While the thermal stability of the first enzyme increased sharply after incorporation in this gel, those of the remaining enzymes remained virtually unchanged when the enzymes were immobilised. In principle, all three enzymes could be stabilised in relation to uncoiling and hence thermal inactivation by forming multipoint hydrophobic contacts with the carrier. However, the enzyme–matrix interaction can be thermodynamically favourable, as shown by the examination of the above mechanism, only subject to the condition that the molecular mobility of the protein molecules

incorporated mechanically in the gel is reduced even in the initial stage. In the gel silastic at a given concentration the inhibition of translational diffusion (and hence stabilisation) is apparently achieved only for very large acetylcholinesterase molecules but not for the relatively small glucose oxidase and peroxidase molecules.

Further prospects. One can hardly give preference to any particular physical immobilisation method without knowing the purpose of the resulting enzyme preparation. If, however, one endeavours merely to increase the thermal stability of the enzyme as a result of immobilisation, then we believe that mechanical incorporation of the enzyme in a gel capable of specific interaction with the protein is most attractive. The advances achieved in this field (see above) can be enhanced still further if an attempt is made to optimise the gel structure using more than one monomer (not counting the cross-linking agent) and thus introducing various functional groups into the carrier[123,133].

On the other hand, one should recall that the stabilisation effect in such systems depends radically on the concentration (density) of the gel (Fig.5) and that only a concentrated gel exerts a stabilising influence (if such an influence occurs at all). Consequently the swelling of gel particles (grains) in water results in the reversible disappearance of the stabilisation effect[119]. It would seem that this limits the applicability of such systems in the formation of stabilised enzyme preparations. However, the swelling of the gel in water can be prevented (and the thermal stabilisation effect can thus be retained) by employing gels which are sufficently cross-linked (by carrying out the cross-linking either directly during polymerisation using comonomers of the type *N,N'*-methylenebisacrylamide or by an additional treatment of the gel particles—grains several microns in size—with bifunctional cross-linking agents)[134]

Thermally stabilised enzyme preparations based on gels have found applications also in the catalysis of reactions in organic solvents. However in this case the organic solvent should be immiscible with water and the gel will hardly swell (after a suitable choice of polymer); consequently the thermal stabilisation effect (of the kind illustrated in Fig.5) should be preserved; the enzymic reactions in water-organic solvent systems will be described in greater detail below.

Biological aspects (model of biological membranes). The mechanism of the stabilisation of enzymes incorporated mechanically in concentrated polymeric matrices (i.e. the forced arrest of the translational movement of the enzyme molecule followed by multipoint interaction between the protein and the relatively immobile matrix), proposed by Martinek et al.[119], can function *in vivo* in membrane enzyme systems[135,136], where, firstly, an extremely low mobility of the molecules in the membrane is observed (the translational and rotational movements are inhibited)[137] and, secondly, the enzyme molecules can interact with the constituent phospholipids and structural proteins in the membrane, forming a set of relatively weak bonds—hydrophobic, electrostatic, hydrogen, etc.[138] Furthermore, such formal similarity makes it possible to understand certain aspects of the functioning of biological membranes. Thus it is known that, in the adaptation of organisms to changes in the temperature of the environment, the thermal stability of membrane enzymes also changes[54]. The molecular mechanism of such regulation may consist in an alteration of the fraction of unsaturated lipids in membranes[54], which should influence the viscosity of the membrane phase and hence the molecular mobility of the enzyme molecules incorporated in it. The molecular mobility and the thermal stability of the enzymes incorporated mechanically in the matrix are in their turn interrelated; this has been shown[119] by the model system "α-chymotrypsin in a polymethacrylate gel" (see above).

4. Mechanical Incorporation of Enzymes in the "Narrow" Pores of the Carrier

The uncoiling of the protein globule on denaturation can also be prevented by accommodating the native enzyme molecule in a cell which does not react with it via either chemical or sorption mechanisms but is so "narrow" that the uncoiled conformation cannot be formed for steric reasons. In this case the native conformation of the protein globule can be maintained by purely mechanical means. This mechanism has been frequently proposed in the literature as a hypothesis (frequently in a very implicit form)[139-142], but experimental evidence has been obtained only recently[134,143,144] in studies of the properties of α-chymotrypsin and trypsin in a polyacrylamide gel or in (on) polysaccharide carriers.

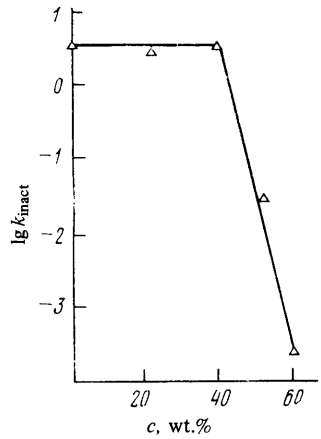


Figure 6. Dependence of the pseudo-first-order rate constant (k_{inact}, min^{-1}), characterising the unimolecular thermal inactivation of α-chymotrypsin incorporated mechanically in a polyacrylamide gel, on the concentration c of polyacrylamide in the gel. Conditions: pH 8, (0.02 M tris.HCl + 0.1 M KCl), 60 °C.[134,143]

The polyacrylamide gel is an extremely suitable carrier for research of this kind: firstly, this gel is chemically inert[145], secondly, the size of its pores can be readily varied by altering the monomer concentration[146], and, thirdly, at fairly high gel concentrations the size of its pores is comparable to the size of enzyme globules[147,148].

Two methods have been used for the mechanical incorporation of the enzyme in the gel: acrylamide at the required concentration is polymerised directly in the enzyme solution (as described by Martinek and coworkers[114,119]) or the gel particles (obtained in the absence of the enzyme and carefully washed free of low-molecular-weight impurities) are impregnated with the enzyme solution and are then lyophilised to give the required concentration of polyacrylamide[143,144]. The thermal stability of the enzyme is the same in both cases (Fig.6): whereas at a not unduly high gel concentration (less than 45 wt.%) there is no appreciable stabilisation of the enzyme in relation to irreversible unimolecular thermal

inactivation, in a denser carrier (more than 50 wt.% of polyacrylamide) the thermal stability of the enzyme increases sharply.

The magnitude of the stabilisation effect. The thermal stability of the enzyme incorporated mechanically in a very concentrated polyacrylamide gel (lyophilised powder) is so much higher than the thermal stability of the enzyme in solution that the two quantities cannot be measured at the same temperature. It is necessary to investigate the temperature variation of the rate of unimolecular thermal inactivation: linear extrapolation of the data presented in Fig.7 to the centre of the temperature range (120 °C, vertical dashed line) shows that the stabilisation effect in a fully dried gel reaches a value corresponding to an increase by a factor of 10^{13}. For less concentrated gels, the stabilisation effect is naturally smaller.

The stabilisation mechanism is not associated with the influence of the non-polymerised acrylamide[149] because it was completely washed out of the comminuted gel[143,144]. Nor can stabilisation be explained by the chemical grafting of the enzyme to the gel, because both chymotrypsin and trypsin could be completely eluted from the swollen gel[143,144]. The enzyme hardly interacts with the matrix via a sorption mechanism, because the protein globule retains a high rotational mobility up to a gel concentration of 50 wt.% (where appreciable thermal stabilisation is already observed)[119,143,144]. Consequently the pore size presumably diminishes with increasing gel concentration and ultimately the enzyme globule is "clamped" and cannot uncoil (be denatured) as a result of the "cage effect".

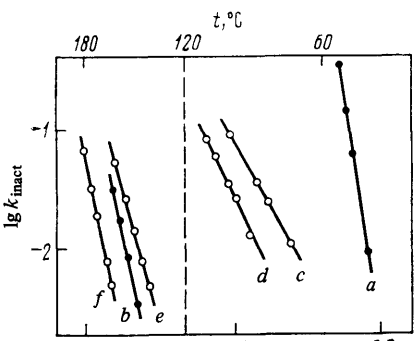


Figure 7. Temperature variation of the pseudo-first-order rate constant (k_{inact}, min^{-1}) characterising the unimolecular thermal inactivation of α-chymotrypsin: *a*) solution of native enzyme (pH 5–8); *b*) lyophilised enzyme; *c*)–*f*) enzyme incorporated mechanically in 48, 60, 80, and 100 wt.% polyacrylamide gel respectively (the polymer concentrations indicated are those in 60 μm gel grains washed after lyophilisation)[134,143].

It might have been supposed that a different stabilisation mechanism operates, namely that the dehydration of the protein can lead to a decrease of the mobility of the polypeptide chains, which would hinder the uncoiling of the globule. A choice between these alternative mechanisms cannot be made in a study of the thermal stability of a lyophilised preparation of the free enzyme. In this state the protein molecules are in all probability involved in a very close packing, where each globule can be incorporated (as in immobilisation in a gel) in a "narrow pore" formed by similar globules. The exceptionally high thermal stability of dry (lyophilised) enzyme preparations[139,141,142] (particularly that of chymotrypsin, see Fig.7), observed experimentally, can be caused both by the "dehydration of the polypeptide chains" and by the "cage effect". This problem was resolved, i.e. an unambiguous choice was made between these two mechanisms, by employing polysaccharide carriers.

Polysaccharide carriers are inert and hardly interact with proteins. This is indicated by the following fact: although the translational diffusion of protein molecules (lactoglobulins, ribonuclease, etc.) virtually disappears in a 40% dextran solution, their rotational diffusion is not significantly inhibited (compared with an aqueous solution without a polysaccharide)[150].

Pseudo-first-order rate constants (min^{-1}) for the irreversible unimolecular thermal inactivation (60 °C) of native and immobilised α-chymotrypsin[134,144].

Preparation	Free enzyme	Enzyme attached to the surface of cellulose	Enzyme incorporated in the pores of Sephadex
Solution or suspension at pH 8 (2×10^{-2} M tris-HCl + 0.1 M KCl)	3	0.1	0.08
Lyophilised state	*	0.05	*

*Thermal inactivation was not observed for 30 min even at a higher temperature (100 °C).

In order to obtain an answer to the above question, a study was made of the thermal stability of chymotrypsin attached covalently to two polysaccharide carriers—cryptocrystalline cellulose and Sephadex. Although these carriers are very similar in their chemical compositions, they are structurally fundamentally different: in contrast to cellulose, Sephadex has a porous structure. Consequently, whereas in the case of cellulose the enzyme is attached to the surface, in Sephadex the attached protein molecule is incorporated within a carrier pore. On lyophilising the carrier, the "cage effect" can therefore obtain in the latter case (and only in the latter case). The Table shows that covalent immobilisation has only a slight influence on the thermal stability of chymotrypsin on both polysaccharide carriers (in relation to the free enzyme). The additional lyophilisation of the enzyme attached to the surface of cryptocrystalline cellulose also does not alter significantly the thermal stability of the enzyme preparation. On the other hand, thermal inactivation of the enzyme attached to Sephadex G-150 could be observed neither under comparable conditions (see Table) nor even at 100 °C (incubation time 30 min).

Since the only significant difference between these two polysaccharide carriers is that in the Sephadex the enzyme was located in a narrow pore after drying, while in cellulose it remained on the surface of the particle, one may reach the following conclusion: the very high increase of the thermal stability of enzymes in concentrated porous carriers (Sephadex and polyacrylamide) is caused by the "cage effect" and not the dehydration of the protein.

Further prospects. Preparations with such high thermal stability can be used in practice if the swelling of the carrier in water is prevented. This can be achieved by employing sufficiently cross-linked polymeric materials or by carrying out enzymic reactions in organic solvents immiscible with water (for further details, see below).

The method involving the immobilisation of protein globules in the narrow pores of an inert carrier leads to new possibilities for the stabilisation (as a result of the "cage effect") of even extremely labile enzymes, which are usually inactivated as a result of immobilisation on interaction with carriers.

In nature a similar mechanism (based on the "cage effect") can stabilise proteins in spores in relation to unfavourable conditions in the environment.

5. Approaches Based on the pH Dependence of the Rate of Thermal Inactivation

The rate of thermal inactivation sometimes depends significantly on the pH (see, for example, Goldstein and coworkers[6,101]). In this case the denaturation of the enzymes can be suppressed by procedures based on the physicochemical principles described below in Sections IV.2 and IV.3.

IV. THE STABILISATION OF ENZYMES IN RELATION TO INACTIVATION UNDER THE INFLUENCE OF EXTREME pH VALUES

Many enzymes lose their catalytic activity rapidly and irreversibly under the influence of what for them are extreme pH values (i.e. at pH values of the medium remote from the natural pH range of their activity)[52]. The "alkaline" inactivation of pig pepsin (which begins already at pH 5) and the "acid" inactivation of chicken lactate dehydrogenase (which takes place effectively at pH 4) can be pointed out as thoroughly investigated examples.

In principle, when an enzyme is immobilised, its pH stability can change (as well as its thermal stability—see the previous Section). Indeed this phenomenon has been observed experimentally[5,6,42,151] for many enzymes. In order to account for it, one usually points (see, for example, Goldstein and coworkers[6]) to the change in the microenvironment of the enzyme when it is immobilised; another possible effective factor may be a change in the enzyme conformation on immobilisation (induced, for example, by its chemical modification or interaction with the carrier).

However, as in the consideration of the thermal stability of enzymes, we are forced to conclude that, despite the probable validity of these causes, they cannot be used in the development of general principles for increasing the pH stability of enzymes, since it is not known in what way the stability is related to the microenvironment of enzymes and their conformations. On the other hand, one can now already formulate several general principles (unrelated to those discussed above), which increase the pH stability of enzymes as a result of their immobilisation.

1. Prevention of the Uncoiling of Enzyme Molecules

The pH inactivation of enzymes, like thermal inactivation, apparently includes as a significant stage the uncoiling of protein molecules[53] caused by the change in the balance of electrostatic and hydrogen bonds when the ionised state of ionogenic protein groups is altered by the change of pH. In this case all the recommendations made in the discussion of the stabilisation of enzymes in relation to thermal inactivation are applicable (see above).

2. Immobilisation of the Enzyme on a Carrier with Buffer Properties

Suppose that the enzyme is immobilised on a carrier with ionogenic groups exhibiting buffer properties with a considerable capacity. It is then evident that, following a change in the pH of the solution, the change in "micro-pH" in the carrier (i.e. the pH values around the enzyme) will always be considerably smaller. This increases the observed pH stability of the immobilised enzyme compared with its native state.

A brilliant illustration of this postulate is apparently provided by the work of Kaplan and coworkers[152], who investigated the acid inactivation of native lactate dehydrogenase and the enzyme attached covalently to glass. Whereas the native enzyme is almost completely inactivated after 1 h at pH 3.2 (25 °C), the immobilised enzyme retains virtually 100% activity under these conditions even after incubation for 35 days (Fig.8). The buffer properties of the carrier surface are in this case confirmed by the fact that, in contrast to the native lactate dehydrogenase, the catalytic activity of the enzyme attached to glass is almost independent of the pH of the medium[152].

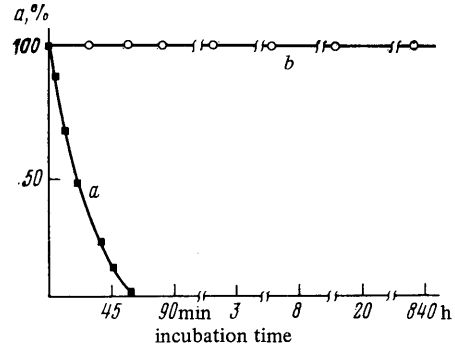


Figure 8. The pH stability of native lactate dehydrogenase (*a*) and the enzyme bound covalently to porous glass (*b*) (25 °C, pH 3.2).[152]

3. The Shift of the pH Profile for the pH Inactivation of the Enzyme

After the studies by Katchalski and coworkers[153,154], which have become classical, the shift of the pH profile of the catalytic activity of enzymes when they are attached to polyelectrolytes appears quite natural[65]. When enzymes are immobilised on polyanions, the pH profile of their activity shifts to the alkaline region (in consequence of the higher proton concentration in the carrier phase compared with the solution), and when they are immobilised on polycations there is a shift to the acid region (in consequence of the lower proton concentration in the carrier phase compared with the solution); see, for example, Fig.9*a*. The shift of the pH

profile of the catalytic activity of enzymes on immobilisation should also be observed, generally speaking, in the case of a non-electrolyte carrier owing to a specific distribution of protons between the latter and the medium[65].

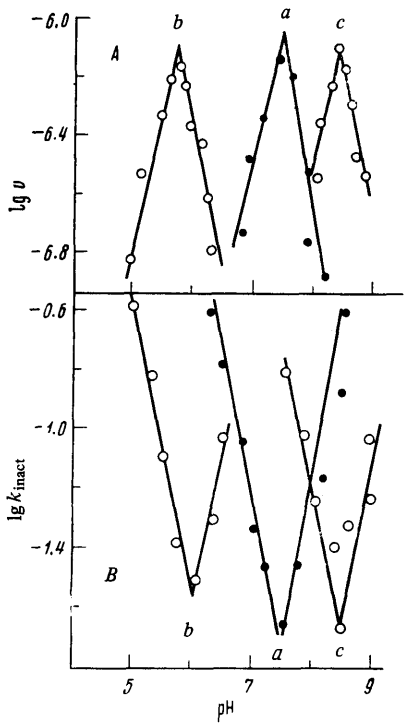


Figure 9. The pH variation of the maximum rate V (arb. units) of hydrolysis of benzylpenicillin catalysed by penicillin amidase (A) and of rate constant (min^{-1}) for the inactivation (at 50 °C) of penicillin amidase (B): a) native enzyme; b) enzyme–polycation complex; c) enzyme–polyanion complex[155].

Evidently analogous effect should obtain also in the pH inactivation of enzymes—the difference between the pH values of the solution and the carrier phase should lead to a shift of the observed pH profile for the pH inactivation process. Indeed, a series of studies by Katchalski and coworkers (see the references in a paper of Goldman et al.[6]) showed that, as was to be expected, the attachment of enzymes to a polyanionic carrier increases their stability at alkaline pH, while attachment to a polycationic carrier makes them more stable in acid solutions. This phenomenon is illustrated by the results of a study[155] (Fig.9) in which the activity and stability of penicillin amidase immobilised on different carriers were investgiated. Evidently (Fig.9B) the pH profiles of the rate constant for the pH-inactivation of the enzyme are displaced depending on the nature of the carrier, in full agreement with this theory.

V. ENZYMIC REACTIONS IN ORGANIC SOLVENTS WITH A LOW CONTENT OF WATER (OR IN ITS ABSENCE)

Many enzymes have been adapted by nature to function effectively primarily in aqueous solutions. This fact limits, in principle, the activation of enzymes as catalysts of reactions employed in practice. The point is that many chemical reactions are thermodynamically favourable only in specific organic solvents, which is due to specific solvation effects, the solubility of individual reactants, and the fact that, apart from the required product, water is sometimes formed, so that the equilibrium is strongly displaced towards the initial substances when the reactions are carried out in aqueous solutions. The last cause inhibits the use of biocatalysis in important processes such as the synthesis of esters or amides, the polymerisation of aminoacids or sugars, dehydation reactions, etc.

Evidently this difficulty can be overcome by replacing water as the reaction medium by a non-aqueous solvent. There have been fairly numerous studies in which attempts were made to carry out enzymic reactions in organic solvents or aqueous organic media with a high concentration of the non-aqueous component[58,156–181]. However, all valid studies of this kind, led to the conclusion that on passing from water to the organic solvent, the catalytic activity of the enzymes falls sharply and their substrate specificity disappears.

A partial solution to this problem can be found by selecting an organic component which denatures the enzyme to the smallest extent[181]. However, two other approaches are more promising: the use of two-phase aqueous organic systems (mixtures of water and organic solvents with which it is immiscible)[59,182] or inclusion of enzymes in "inverted" micelles of surfactants[183]. We shall consider in greater detail the physicochemical principles of these three approaches.

1. Criteria for the Selection of the Optimum Organic Solvent

In order to carry out enzymic processes in non-aqueous or aqueous organic media with a high content of the non-aqueous component, one uses as a rule organic solvents such as dimethyl sulphoxide[158–165], formamide[163,166], dimethylformamide[163,167–169], dioxan[170–174], ethylene glycol[172,175], glycerol[175,176], monohydric alcohols[165,172,177–180], and acetone[165,174]. The choice of a particular solvent is in most cases random and lacks theoretical foundations.

The criterion for the selection of the optimum solvent (from the standpoint of its influence on the enzyme) can be based on the general ideas of the relative role of the forces maintaining the native structure of protein molecules in aqueous solutions: hydrophobic interactions play a dominant role[79]. Consequently a non-aqueous solvent which is "satisfactory" for the protein should not disrupt such interactions.

In order to obtain an answer to the question which solvents have this property, we shall turn to data for the model process involving the formation of surfactant micelle in various solvents. Ray[184] concluded that hydrophobic interactions constitute merely a special case of solvophobic interactions. All solvents can be divided into three classes in terms of their ability to undergo solvophobic interactions: the first class includes water, glycerol, ethylene glycol, aminoethanol, formamide, and certain others; the second includes methyl, and dimethyl-formamides, and the third includes methanol, ethanol, and toluene. Solvophobic interactions occur to the greatest extents in solvents of the first class (thus it has been found that[184] detergent micelles are formed only in such solvents); they take place to a much lesser extent in solvents

of the second class and are virtually absent in solvents of the third class.

According to Ray[184], this difference is due to the fact that the molecules of solvents of the first class contain at least two or three fragments capable of forming hydrogen bonds. As a result of intermolecular interactions, hydrogen bonds form rigid (thermodynamically unfavourable) skeletal solvent structures around the solvophobic solute molecules. This leads to solvophobic (or in the special case hydrophobic interactions). In the series of solvents of the first class solvophobic interactions occur most effectively in water and glycerol (estimated from critical concentrations for micelle formation); in the remaining solvents this ability is significantly smaller.

The above data made it possible to formulate[181] a criterion for the choice of the optimum solvent. The criterion is extremely universal, because the efficiency of solvophobic (as well as hydrophobic[185]) interactions is determined primarily by the nature of the solvent and not the nature of the interacting molecules. Consequently if it is assumed that hydrophobic interactions play the main role in maintaining the structure of protein molecules[79], one must recognise that the best solvents for enzymic reactions should be those of the first class, including water (which is employed in nature) and glycerol.

We may note that the authors of the majority of studies used solvents belonging to the second or even third group (see above), i.e. the least successful solvents. However, in some investigations it was nevertheless found experimentally[172,180,186,187] that solvents belonging to the first class (particularly glycerol and to a lesser extent ethylene glycol) denature proteins much less than others. These empirical findings can be given a physicochemical explanation in the light of studies by Martinek and coworkers[181,184].

2. Enzymic Reactions in Two-Phase Systems Comprising Water and an Organic Solvent Immiscible with It

Following the development of research into immobilised enzymes, studies began to appear whose authors attempted to make enzymes function in organic solvents and aqueous organic media with a high concentration of the organic component having increased their stability in relation to the denaturing influence of the non-aqueous medium by attaching them to a carrier (see, for example, Refs.165, 168, 169, 173, 174, 176, 179, 180, and 189–191). However, even in the most successful systems, when the concentrations of the organic component are above 90%, the immobilised enzyme is nevertheless inactivated. Thus, from the standpoint of preparative enzymic synthesis, the problem formulated remains unsolved. It is extremely likely that in this form it is insoluble, in principle, because, on passing from water to a non-aqueous medium, there is bound to be a significant change in the conformation of enzymes[58,192] and a consequent sharp impairment of their catalytic function.

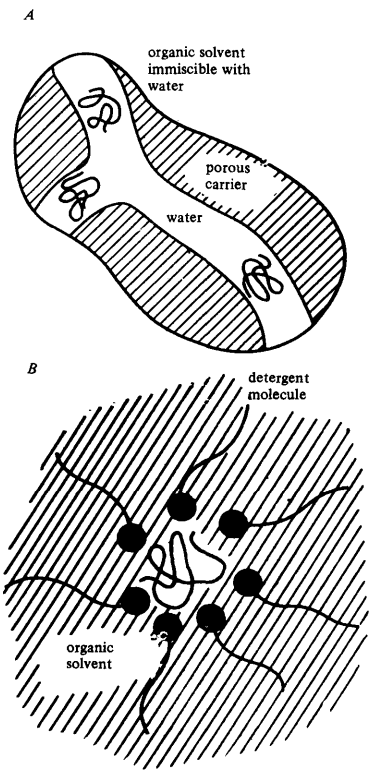
We found a fundamentally new solution[59,182]. In all the studies with which we are familiar, where the behaviour of enzymes was investigated in aqueous organic or organic media, solvents such as acetone, acetonitrile, dioxan, dimethyl sulphoxide, dimethylformamide, methanol, ethanol, and others (see above) were used as the non-aqueous component. A common feature of these solvents is that they are infinitely miscible with water. This is in fact why one has to solve the problem of the stabilisation of enzymes in relation to inactivation by the organic component.

The basic idea of our approach[59,182] is that the organic solvents added to the enzyme-containing aqueous phase should be virtually immiscible with water (for example, chloroform, ether, aliphatic alcohols, hydrocarbons, etc.). In this case the resulting system will consist of two phases—aqueous and organic. The enzyme will be contained solely in the aqueous phase, because, firstly, it can be immobilised there, and, secondly, even if immobilisation is impossible, proteins, which are satisfactorily soluble in water, are usually virtually insoluble in hydrophobic solvents[58]. The substrates, being soluble in the organic phase, can in their turn diffuse freely[193,194] from the latter to water, where they undergo a chemical transformation under the influence of the enzyme; the products diffuse from water back to the organic phase. Thus the thermodynamic equilibrium in the given chemical reaction is established throughout the aqueous organic system via water (with the aid of the enzyme catalyst present in water).

Such a two-phase system has a number of fundamentally important properties: (1) since the enzyme is not in contact with the non-aqueous component of the reaction medium, there is no need to solve the problem of its stabilisation[59,192]; (2) the volume fraction of the organic phase can be in principle as close to unity as required, so that equilibrium conditions in a two-phase system of this kind can be made as close to equilibrium in the pure organic medium as one wishes (see the theoretical aspects below[59,182]); (3) a high content of the non-aqueous component of the medium can ensure also a solubility of hydrophobic reactants, for example, steroids, sufficient for preparative synthesis[195,196].

Methodological aspects. The enzyme system comprising water and an organic solvent immiscible with it can be prepared as an emulsion of an aqueous solution of the enzyme in the organic medium or as a suspension in the organic medium of porous particles (for example, a porous glass or ceramic, a hydrophilic gel, etc.) impregnated by the aqueous enzyme solution[59,182] (see Scheme 2, *A*), the latter procedure being much more convenient both methodogically and tecnologically.

Scheme 2



The aqueous enzyme solution can also be enclosed in microcapsules or hollow fibres[9,11] or even in the microcracks of a polymeric carrier by mechanical deformation of the latter[197]. The enzyme can be used in both free and immobilised states.

It would seem that preparative enzymic synthesis in a two-phase system is inapplicable to reactions in which the reactants (and in the first place products) are ions, because ions cannot pass from water to the organic phase. However this difficulty can be overcome[182] by selecting hydrophobic counterions for ionic reactants (as suggested in the chemistry of nucleotides[198] and used widely in phase transfer catalysis—see, for example, Dehmlow[199]).

Examples. (a) The idea of carrying out enzymic organic synthesis in a two-phase aqueous organic medium with a high content of the non-aqueous component was tested[59,182] in relation to the chymotrypsin-catalysed synthesis of the methyl ester of *N*-acetyl-L-tryptophan from ethanol and *N*-acetyl-L-tryptophan. Since water is formed together with the ester in the synthesis, the equilibrium was strongly displaced towards the initial reactants when the reaction was carried out in water (see the references in the papers by Martinek et al.[200] and Carpenter[201]). Furthermore, at neutral pH (at which the enzyme employed is active) there is an additional shift of the equilibrium unfavourable for the synthesis of the ester and caused by the thermodynamically favourable ionisation of one of the initial components—the acid. Ultimately the yield of the ester is negligible, amounting to less than 0.01% even at a relatively high ethanol concentration (~10 M). In the "chloroform + 1 vol.% of water" two-phase system the equilibrium was completely displaced towards the ester[59,182] and the yield was virtually 100%.

The enzymic synthesis was carried out as follows. The porous glass was simply soaked in an aqueous buffer solution (1 M KH_2PO_4, pH 7) of chymotrypsin (5×10^{-4} M) and then 0.3 g of the resulting preparation was transferred to 30 ml of chloroform containing the substrates, i.e. 10^{-2} M *N*-acetyl-L-tryptophan and 1 M ethanol, and the system was stirred overnight. Analysis of the resulting mixtures showed that the entire *N*-acetyl-L-tryptophan was converted into the corresponding ethyl ester. The identity of the product with the commercial ethyl ester of *N*-acetyl-L-tryptophan was demonstrated by gas–liquid chromatography—the retention times agreed. In order to isolate the product, it was sufficient to separate the aqueous phase and to evaporate the chloroform on a rotating evaporator.

(b) The equilibrium could be shifted analogously in the synthesis of glycerophosphate from glycerol and inorganic phosphate catalysed by alkaline phosphatase[182]. In order to ensure the solubility of the ionic phosphate reactants in the two-phase "chloroform + 1 vol.% of water" system, tetrabutylammonium was used as the counterion. Whereas the yield of the ester in water[188] was neglibile (less than 0.1%), in the two-phase system it amounted to 30%.

Theoretical aspects and prospects. The observed shift of the equilibrium is common to chemical reactions. In order to demonstrate this, consider the reaction

$$A + B \underset{}{\overset{K}{\rightleftharpoons}} C + D, \qquad (1)$$

where K is the equilibrium constant. Suppose that the aim of the synthesis is, for example, the formation of product C. Its yield is evidently defined by the expression

$$[C] = K[A][B]/[D]. \qquad (2)$$

It is seen that there are two causes of the shift of the equilibrium (and of the increase of the yield of product C) on passing from water as the reaction medium to the two-phase aqueous organic system described. Firstly, if the product D of the reaction is water, a decrease of its concentration in the system leads to an increase of the yield of products in accordance with Eqn.(2). Thus in the syntheses described above a change from 100% water to an approximately 1% aqueous system resulted in an approximately 100-fold increase of the yield of the ester.

Secondly, the shift of the equilibrium in reaction (1) can occur due to a change in the effective equilibrium constant on passing from water to the two-phase system. Suppose that the equilibrium constant for reaction (1) in water is K_w. Then the effective equilibrium constant in the two-phase system K is defined (by analogy with the study of Martinek et al.[202]) by the expression

$$K = K_w \frac{(1+\alpha P_C)(1+\alpha P_D)}{(1+\alpha P_A)(1+\alpha P_B)}, \qquad (3)$$

where α is the ratio of the voumes of the non-aqueous and aqueous phases and P_A, P_B, P_C, and P_D are the partition coefficients of the reactants between the non-aqueous and aqueous phases. We shall assume that the initial reactants A and B are sparingly soluble in the organic phase, but are effectively soluble in water, i.e. have low partition coefficients—for example, $P_A \cong P_B \cong 10^{-2}$. In this case, even if only one of the products is sparingly soluble in water but effectively soluble in the organic phase, i.e. has a high partition coefficient, for example, $P_C \cong 100$, then it follows from Eqn.(3) that $K > 2500\ K_w$ for $\alpha = 100$.

Evidently the effective equilibrium constant in the two-phase system can in principle exceed that in water by many orders of magnitude. This constitutes an additional (in some cases the main one) cause of the shift of equilibrium (1).

In principle, by varying the nature of the non-aqueous phase (and hence by varying the partition coefficients of the reactants), it is possible to alter K as required and hence also the yield of the product. The partition coefficients of ionic reactants can be altered (and hence the reaction equilibrium can be displaced in the required direction) by selecting hydrophobic counterions (see the methodological aspects described above).

3. Catalysis by Water-Soluble Enzymes Incorporated in "Inverted" Surfactant Micelles in Organic Solvents

The molecules of many surfactants form in organic solvents associated species of the type of "inverted" micelles in which the polar (ionic) groups of the surfactant molecule constitute the nucleus of the associated species, while the hydrocarbon fragments make up the outer layer[203–205]. Such micelles are known to be capable of solubilising ions, polar organic substances, as well as considerable amounts of water (several tens of H_2O molecules per surfactant molecule). It has been shown recently[183] that relatively high concentrations of biopolymers (enzymes) can also be solubilised by micelle-forming surfactants in organic solvents, up to 1 mg ml^{-1}, which corresponds to $\geqslant 10^{-5}$ M active centres (for a molecular weight of the enzyme not more than 100 000) and greatly exceeds the required level of "catalytic concentrations" of the majority of enzymes[1,2]. The enzyme then retains almost completely its catalytic activity and substrate specificity.

When it is enclosed in the inverted micelle (see Scheme 2, *B*), the enzyme molecule is apparently protected against denaturation (against the uncoiling of its structure) by the fact that the "interface" between the protein globule (or its surface layer of water) and the organic solvent is stabilised by the surfactant molecules. Ultimately the biocatalyst may not in fact come into direct contact with the organic solvent unfavourable for it, being located in a kind of microreactor

containing only several hundredths of H_2O molecules per enzyme molecule (this corresponds to a total water content in the organic solvent–surfactant system less than 1 vol.%). Thus the relative catalytic activity of α-chymotrypsin solubilised in octane is retained for months at room temperature[183].

The systems investigated. All these postulates have been demonstrated[183] in relation to the proteolytic enzyme chymotrypsin (in the hydrolysis of the *p*-nitroanilide of *N*-glutaryl-L-phenylalanine) as well as peroxidase [in the oxidation of hexacyanoferrate(II) ions and pyrogallol]. The reactions of these enzymes with their specific substrates have been investigated in extremely hydrophobic (hydrocarbon) solvents, namely octane and benzene, using as the surfactant a product manufactured on a large scale—the di-(2-ethyl) ester of the sodium salt of sulphosuccinic acid (Aerosol OT), which was kindly supplied to us by Prof.Menger (USA). Data for the formation and properties of the micelles of this surfactant in organic solvents have been reviewed[206]. A typical experiment was as follows: 2 ml of 0.1 M solution of the surfactant in the hydrocarbon was treated with $\leqslant$0.01 ml of a concentrated (10^{-3} M or less) solution of the enzyme in an aqueous buffer, $\leqslant$0.01 ml of the substrate solution in water or acetonitrile was introduced, and the rate of enzymic reaction was measured spectrophotometrically in the resulting homogeneous (optically transparent) system.

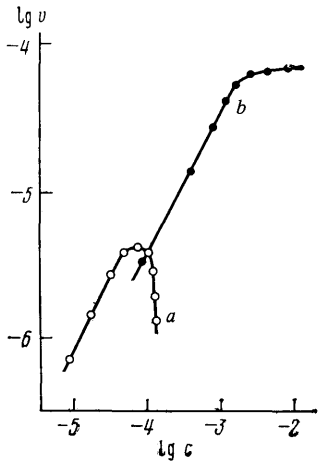


Figure 10. Dependence of the initial rate of the peroxidase oxidation of pyrogallol in water (*a*) and octane (*b*) on the molar pyrogallol concentration. Conditions: pH 7, 26 °C, 2.5×10^{-3} M H_2O_2, 3×10^{-9} M peroxidase, in octane containing 2 vol.% of water and 0.01 M Aerosol OT.[183]

As an example, Fig.10 presents data[183] for the peroxidase oxidation of pyrogallol. Evidently, when the reaction is transferred from water to a medium containing inverted micelles in octane, the kinetic mechanism of the catalysis changes. This is associated with the fact that in the latter case there is no substrate inhibition characteristic of the reaction in water.

Use in fundamental biochemical research. Enzymes solubilised by the method of Martinek et al.[183] in organic solvents (to which a surfactant forming inverted micelles has been added) will undoubtedly find applications as models of enzymes functioning *in vivo* in biological membranes and also in the study of the role of water in the structure of proteins and of the mechanisms of enzyme catalysis (particularly the catalysis of processes where water is a chemical reactant). The point is that the amount of water solubilised in the system and hence the amount surrounding the enzyme in the inverted surfactant micelle can be strictly controlled.

VI. CONCLUSION

1. Stabilisation of Enzymes under the Influence of Water-Soluble Additives

We endeavoured to describe as fully as possible the problem of the stabilisation of enzymes, but we deliberately disregarded an enzyme stabilisation phenomenon which is widespread and is frequently discussed in the literature; it takes place under the influence of effectors such as bivalent metal ions (in the first place Ca^{2+}, Mg^{2+}, Sr^{2+}, Zn^{2+}, Co^{2+}, and Mn^{2+} [207–210]) ammonium sulphate[211], various anions (in the first place phosphate and sulphate[212–215]), serum albumin[216,217], polyols (in the first place glycerol as well as ethylene glycol, sucrose, etc.)[218–220], detergents[221], and organic solvents[222,223].

In the present review we did not consider this type of stabilisation of enzymes for the following reasons. Firstly, the above effects are not general: the same effector can stabilise one enzyme and reduce the stability of another[211,224]. At the same time a given enzyme can be stabilised by one of the above effectors and labilised by others[225]. Secondly, in most cases the physicochemical mechanism of the stabilisation of enzymes by the effectors is obscure and in others is trivial—for example, sometimes when serum albumin is added as a stabilising additive. This protein is either effectively adsorbed on the interface (for example on the water/air interface) and thus prevents the surface inactivation of the enzyme itself, or it sorbs heavy metal cations and other admixtures which "poison" the enzyme. At the same time, one must emphasise the practical and theoretical importance of studies in this field, particularly those with the correct quantitative profile.

2. Two General Principles of the Stabilisation of Enzymes

In the present review we considered fairly extensively only the general physicochemical approaches, which are also of technological importance and which protect the enzyme from denaturing factors in the medium (elevated temperature, extreme pH values, and the addition of organic solvents). The entire wide variety of approaches discussed (see Sections III–V) can be divided into only two groups, corresponding to the following two general principles.

(1) An increase of the rigidity of the protein globule which prevents its uncoiling on denaturation and hence inactivation; this can be achieved both by cross-linking the protein (with formation of a water-soluble preparation) and as a result of the multipoint interaction of the protein (both covalent and physical) with a complementary carrier surface (for the preparation of an insoluble heterogeneous catalyst).

(2) The spatial separation of the enzyme and those factors in the medium which do not favour the retention of catalytic activity and specificity. The application of this principle depends on the nature of the inactivating influence. Thus, in order to prevent the pH inactivation, one can immobilise the enzyme on a carrier either having buffer properties or

ensuring a local shift of the pH in its surface layer. The influence of the organic solvent can also be avoided in two ways: either by carrying out the enzymic reaction in a two-phase system comprising water and an organic solvent immiscible with it or by enclosing the enzyme in the "inverted" micelles of a surfactant.

3. Stabilisation of Enzymes as a Problem with Optimum Solution

The methods for the stabilisation of enzymes developed at the present time (in relation to inactivation as a result of the uncoiling of protein globules) can lead in many instances to undesirable secondary effects, which hinders somewhat the practical application of biocatalysis.

Thus, firstly, when attempts are made to stabilise the enzyme, the increase in the rigidity of the protein globule should not be too extreme: otherwise a loss of catalytic activity may ensue. The point is that the functioning of the active centre presupposes as a rule the concerted action of several functional groups of the protein and therefore requires a definite mobility of the polypeptide chains[1,2]. Incidentally, the relation between the stability and catalytic activity of enzymes apparently plays an important role in the adaptation of the organism to changes in the environment (temperature, salt content in sea water)[54].

Secondly, when the enzyme is included in a carrier (particularly in a gel), there may be an increase of steric hindrance to the interaction of the enzyme with macromolecular substrates (see the references in the reviews by Berezin et al.[5,65]).

Thirdly, and finally, in unduly dense carriers, which ensure a high thermal stability of the immobilised enzyme, there may arise in their turn significant diffusion-dependent obstacles to the exchange of reactants between the catalyst and the medium (see the references in Refs.5 and 64–66).

Consequently, in many instances enzyme stabilisation involves a problem with an optimum solution, i.e. it is necessary to optimise the enzyme immobilisation process in such a way that the gain in thermal stability is achieved at the expense of only a slight loss of catalytic activity.

Naturally, approaches based on the principle of the spatial separation of the enzyme and those factors in the region which do not favour its functioning are largely or completely free from these disadvantages.

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