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Kinetic Aspects of the Irreversible Thermal Inactivation of Enzymes

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The methods for the kinetic description and the mechanisms of the thermal inactivation of proteins (enzymes) in various media (aqueous solutions, inverted surfactant micelles, and microsomal membranes) are examined critically. The effective kinetic constants calculated from a first order equation and by approximate methods in conformity with an associative-dissociative mechanism are compared. The temperature dependence of the effective inactivation rate constants is analysed for enzymes and the causes of its deviation from the Arrhenius equation are discussed. The influence of polyols and detergents on the stability of enzymes and their modified forms is analysed and the thermodynamic activation parameters (ΔH^{*}, ΔS^{*}, and ΔG^{*}) for the thermal inactivation of different enzymes in various media are compared and discussed. Recommendations are given concerning the quantitative description of the thermal stability of proteins (enzymes) under the real conditions of their application.
The bibliography includes 138 references.

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I. INTRODUCTION

Major advances have now been achieved in the study of the structure and the principles governing the structural organisation of proteins,[1] but the problem of establishing the interrelation between the structure and stability of biomacromolecules remains one of the unsolved problems in modern biophysical chemistry.[2] It is well known that the changes in the conformation of proteins affect their spectroscopic and many other physicochemical characteristics.[1-3] Sometimes these characteristics fail to "notice" small and only at first sight insignificant conformational transformations of proteins. Slight changes in the structure of the active centre of the biomacromolecule fairly frequently have a significant influence on its stability and functional activity. The study of the loss of stability and activity by proteins is of fundamental importance for modern enzymology, bioorganic chemistry, and related disciplines. On the other hand, unless the principal structural factors determining the stability of protein molecules in media of different nature are known, it is impossible to solve practical problems concerning the stabilisation of biologically important polymers. The necessity for the solution of this problem is dictated by the development of modern biotechnology and engineering enzymology, which require the carrying out of many enzymic processes under severe conditions, i.e. at elevated temperatures, under the influence of mixtures of organic solvents, in aqueous media with extreme pH values, in the presence of detergents of different nature, etc.

Modern biotechnology requires accurate quantitative characteristics of the stability of biopolymers. Numerous data obtained under different conditions frequently cannot be compared let alone used in calculations for enzyme reactors, the permitted usage times of biopreparations, etc. because of the lack of a quantitative treatment. The quantitative characteristics of the stability of biopolymers are especially important under the conditions of their practical application, i.e. in aqueous media, in the presence of organic solvents, detergents, denaturing additives, and stabilising substances, and in the immobilised state.

The interest in the inactivation and reactivation of proteins and enzymes was manifested very long ago[4,5] and this multiaspect problem still remains in the centre of attention of specialists in various disciplines.[3,6-23] Various aspects of the denaturation and inactivation of biopolymers have been examined in monographs,[3,7-11,14,16,18] review articles,[12,13,15,17,21,22] and textbooks.[1,2] The structure of proteins and the role of conformational changes in their denaturation processes have been described in detail in several books.[1-3,9-11] The thermodynamics of the reversible and irreversible denaturation processes have been considered in a number of communications.[3,7,12] The methods for the investigation of the denaturation processes involving proteins and enzymes have been described in detail in the books by Joly[3] and Kushner.[11] The biological aspects of the stability of multienzyme complexes and other biopolymers have been dealt with by Kushner and the kinetic aspects of this problem have been considered in the monograph of Berezin and Varfolomeev.[16] The principal ways of stabilising enzymes have been examined in a number of communications[13-15,19,22] and two reviews[20,21] have been devoted to problems of the reactivation of biocatalysts. The general characteristics of the changes in the thermal stability of proteins and enzymes after the chemical modification of their functional groups have been considered in a review[22] and the relative contributions of thermodynamic and kinetic factors to the stabilisation of immobilised proteins have been dealt with by Kozlov.[23]

In our view, the extensive but by no means complete literature data mentioned above reflect inadequately and sometimes do not deal with many important kinetic aspects of the irreversible inactivation of enzymes. Regarding the stability of enzymes as a function of their structure, which in many respects depends on the environment, and being guided by the fundamental and practical problems mentioned

above, we had several specific aims in the present review: (1) to examine the methods for the rapid and correct characterisation of the rates of enzyme inactivation processes under the real conditions of the application of enzymes; (2) to characterise quantitatively the thermal stability of a number of enzymes (lysozyme, lactate, malate, and glucose-6-phosphate dehydrogenases, horseradish peroxidase, and catalase) and proteins (myoglobin and cytochrome b_5) by means of these procedures; (3) to compare in relation to specific examples the thermal stabilities of enzymes in aqueous solutions and inverted surfactant micelles in organic solvents (peroxidase, glucose-6-phosphate dehydrogenase, and cytochromes b_5 and P-450) and also in aqueous solutions and in native biological membranes (cytochrome P-450); (4) to analyse the characteristic features of the stabilising action on enzymes by polyols and detergents of different nature; (5) to consider critically the thermodynamic activation characteristics of the irreversible inactivation of proteins under various conditions and the possibility of their relation with the structure of the biopolymers and the environment.

II. METHODS FOR THE KINETIC CHARACTERISATION OF THE INACTIVATION OF PROTEINS

The concept of "inactivation" is frequently incorrectly replaced in the literature by the term "denaturation". Inactivation is always associated with the loss of functional activity by the protein and enzyme and can be due to a series of causes which do not induce the denaturation of the biopolymer. The term "inactivation" is much broader than "denaturation". We shall attempt to define clearly these concepts. Kushner has analysed the different definitions of the denaturation of biopolymers.[11] As a result, he justified what he believed to be the most correct definition of this process, namely that denaturation is a conformational change in the biological macromolecule leading to the irreversible or reversible loss of its ability to perform a specific biological function. Denaturation can be induced by physical and chemical factors (heating, ultrasound, irradiation, extreme pH values) and may accompany protein aggregation processes, the dissociation of the subunit biomacromolecules into monomers, the thiol–disulphide exchange, and the cleavage of S–S bonds and can also be the result of immobilisation and chemical modification of the functional groups of the biopolymer.[3,11,20–23] It is assumed that the denaturation process is not accompanied by the dissociation of covalent bonds in the polymer chains of the biomacromolecule.[1–3,11,13,14] The denaturation of the protein always leads to its inactivation. However, inactivation may be caused by factors which do not entail conformational consequences, i.e. denaturing: for example, the interaction of macromolecules with one another or the modification of functionally important groups which does not alter the conformation of the biopolymer as a whole.[20,21] Thus any change leading to the irreversible or reversible loss of a specific biological function may be regarded as the inactivation of the biomacromolecule.

The reversible "native protein (nat) $\rightleftarrows$ inactivated protein (in)" process can be characterised by the equilibrium constant $K = [in]/[nat]$, where [in] and [nat] are the equilibrium concentrations of the inactivated and native forms respectively. In this case, if the protein is present in a highly dilute solution, the equilibrium constant K can be related to the characteristics of the equilibrium inactivation (the enthalpy ΔH and entropy ΔS) by the expression:

$$-\ln K = \Delta G/RT = \Delta H/RT - \Delta S/R$$

The enthalpy ΔH can be determined from the van't Hoff equation subject to the condition that ΔH and ΔS are independent of temperature:

$$-[d \ln K/d(1/T)] = \Delta H/R$$

The values of ΔH and ΔS have now been measured by various methods for the reversible inactivation processes involving a multiplicity of proteins. In order to determine these thermodynamic characteristics, measurements are made of the equilibrium constant between the native and denatured forms or the heat of denaturation is found by scanning microcalorimetry.[12] Tables giving compilations of these data may be found in monographs and reviews.[1–3,7,10–12]

An experimental criterion in testing for irreversibility is especially important in the study of irreversible protein (enzyme) inactivation processes. Those proteins whose functional activity has changed in such a way that after the removal of the given influence the initial level of functional activity is not restored for a long time should be regarded as irreversibly inactivated.[20] A certain indefiniteness in this concept is associated with the arbitrary choice of the time after which the initial functional activity of the protein is not restored.

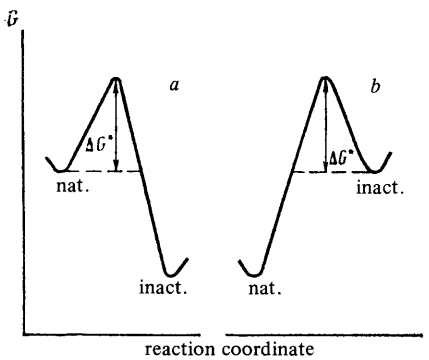


Figure 1. The energy diagram for the possible pathways in the irreversible protein inactivation process: *a*) thermodynamic; *b*) kinetic.[20,21]

The nature of irreversible inactivation can be both thermodynamic and kinetic (Fig. 1).[20,21] In the former case the free energy of the stabilisation of the protein $\Delta G_{in} = G_{in} - G_{nat}$ is greater than zero and the free energy of its destabilisation $\Delta G = -\Delta G_{in} < 0$. The protein cannot be reactivated, since the free energy of the native form is characterised by a local minimum, while the free energy of the inactivated form is characterised by a global minimum (Fig. 1*a*). In the case of the kinetic irreversibility, the protein passes from the native state with a global free energy minimum to the inactivated state with a local minimum in the Gibbs free energy: in this case the free energy of the stabilisation of the protein $\Delta G_{in} < 0$ and the energy of its destabilisation $\Delta G = -\Delta G_{in} > 0$. The two states are separated by a high activation barrier, which prevents the rapid reactivation of the protein (Fig. 1*b*) The practical separation of thermodynamic and kinetic irreversibility in the inactivation of proteins is a complex problem, the ways for the solution of which are only being outlined.[23] The aim of the present review is not the analysis of the contributions to the thermodynamic parameters ΔG, ΔH,

and ΔS by forces of different nature and the examination of data on the reversible inactivation of proteins, since this has already been done in a monograph[3] and other reviews.[12,17,22]

In irreversible inactivation, the kinetic stability of proteins has come to be characterised by the rate constant k_{in}, which is usually determined from the variation of the properties of proteins by fluorescence and electronic and circular dichroism (CD) spectroscopic methods or from the change in activity. In the general case, the retention of the activity of the biocatalyst does not always correspond to the retention of the integrity of the entire protein globule.[22] If one of the postulates of the theory of absolute reaction rates, stating that the rate of attainment of equilibrium between the reactants and the activated complex is much greater than the rate of the chemical reaction itself, is valid, then this theory can be applied to protein inactivation processes.[3,6] According to Eyring,[6] we have the following expression for molecular reactions:

$$k_{in} = (kT/h)\exp(-\Delta G^*/RT) = (kT/h)\, e^{\Delta S^*/R} e^{-\Delta H^*/RT} , \qquad (1)$$

where k is the Boltzman constant, h the Planck constant, and ΔG^*, ΔH^*, and ΔS^* are the increments in the free energy, enthalpy, and entropy on formation of the activated complex. The change in the activation enthalpy is linked to the activation energy by the relation $\Delta H^* = E_{act} - RT$.

However, in the early studies, whose complete survey may be found in a monograph,[3] it was already shown that the irreversible inactivation process can be described by a kinetic first-order equation for by no means all enzymes and not under all conditions. The enzyme inactivation kinetics frequently do not obey the first order law from the beginning of the process or are not described by this law when the inactivation of proteins is continued until the attainment of high degrees of inactivation. The first order equation is usually inapplicable to the description of the inactivation of subunit enzymes in buffer solutions.[24-33] It has been shown[24-32] that, in the inactivation of alkaline phosphatase,[31] kidney acylase,[29] and glucose oxidase,[26,27] these subunit enzymes dissociate into the monomers, which are then irreversibly inactivated. The dissociation of the enzyme into subunits is observed also in the inactivation of glyceraldehyde-3-phosphate dehydrogenase,[34] catalase,[35,36] and many other proteins with a quaternary structure.[20,21,37-39] The appearance of subunits in the inactivation of enzymes has been confirmed by gel-electrophoretic[26,27,29,34] and sedimentation[36] methods. Numerous experimental data have shown that the inactivation of subunit enzymes can be described in the simplest case by the scheme:[24-33]

$$E \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} A \xrightarrow{k_2} I , \qquad (2)$$

where E is the native enzyme, A its reversibly denatured form which partly retains its catalytic activity or a subunit of the oligomeric enzyme, and I the irreversibly inactivated form of the protein lacking enzyme activity. For many enzymes, the inactivation scheme may be much more complex, since the process includes not one but several reversible stages, which greatly hinders its kinetic description. For example, in the presence of Mg^{2+} or Zn^{2+} ions, alkaline phosphatase forms tetramers, which are inactivated via a three-stage dissociative mechanism:[31]

$$E_4 \rightleftarrows 2E_2 \rightleftarrows 4E \rightarrow 4I \qquad (3)$$

In scheme (3), the tetramers E_4 and the dimers E_2 are catalytically active, while the monomers E are inactive and the form I is irreversibly denatured. It has been stated that only the alkaline phosphatase dimers E_2 are catalytically active in the enzymic process.[40] The dissociative mechanism of the inactivation of many subunit proteins is not unexpected, since it reflects their complex structural organisation and the important role which structural levels of different orders play in the functioning of oligomeric protein.

At first sight, it is surprising that comparatively simple monomeric proteins, for example, lysozyme, exhibit a complex inactivation pattern both in solution and in the crystalline state: at pH 3–7 the thermal inactivation of lysozyme is a two-stage reversible process,[41] which corresponds to scheme (2). The stepwise character of the thermal denaturation of lysozyme has been confirmed by the change in the optical density of its solutions in the temperature range 20–90 °C. At 60–70 °C conformational changes take place in lysozyme in solution, accompanied by a decrease of the content of α-helices from 30 to ~10%. Conformational transitions in lysozyme in solution between 20° and 30 °C have been observed also by ^{13}C NMR.[43] The inactivation of lysozyme by ultrasound in the temperature range 20–80 °C showed that the enzyme undergoes several conformational transitions, including one which occurs at ~30 °C.[44] The study of the thermal denaturation of lysozyme in weakly alkaline solutions by fluorescence[45] and circular dichroism[46] methods confirmed the validity of scheme (2) for this enzyme. In the first irreversible stage of the inactivation of lysozyme, the α-helix structure of the protein changes little, but in the second, irreversible stage its complete breakdown is observed over section 5–15 of the polypeptide chain together with the ionisation of the Glu-35 residue, which greatly destabilises the adjoining α-helices. We obtained data indicating the maximum rate of inactivation of the protein in solution at pH 6.5, which confirmed the important role of Glu-35 in the irreversible inactivation of the enzyme,[47] since $pK_a \approx 6.5$ which is characteristic of only the Glu-35 residue localised in the lysozyme molecule in the vicinity of the active centre.[48,49]

We have examined two causes of the deviation of the inactivation of enzymes from the kinetic first-order law—the subunit nature of many proteins and the complex multistage character of the inactivation of monomeric proteins. In a study of the inactivation of enzymes in biological membranes, the process kinetics are complicated still more by the involvement in the process of isoenzymes of the same enzyme or of different conformers of the same form of the protein. This is the situation in the inactivation of cytochrome P-450 in microsomal membranes.[50] The heterogeneity of cytochrome P-450 in liver microsomes leads to a break on the kinetic curves in the semilogarithmic form, reflecting the inactivation of different forms or different conformers of cytochrome P-450 (Fig. 2). In this case the time variation of the concentration of the catalytically active cytochrome is represented by the sum of two exponential functions. The rate constant k_{in}, determined graphically for the first process stage from the semilogarithmic form of the kinetic curve, approaches its true value provided that the rate of inactivation in the second stage is much lower than in the first.

Yet another cause of complication of the kinetics of the inactivation of monomeric proteins is their association (aggregation) in buffer solutions.[3,20,21,51] As a rule, inactivation with formation of aggregates is described by the two-stage kinetic scheme[51] $E \overset{\rightarrow}{\underset{\leftarrow}{\rightleftarrows}} D \rightarrow I$, in which E is the native form, E the denatured form, and I the inactivated form (aggregate). Evidently, this scheme does not differ formally from scheme (2).

Examination of extensive experimental data on the inactivation of monomeric and oligomeric (subunit) proteins led Arens et al.[33] to the conclusion that the inactivation of all proteins can be described by a general formal scheme which is identical with scheme (2). The first reversible stage reflects

the dissociation of subunit proteins and the conformational changes (denaturation) in monomeric proteins. The presence of the first reversible stage leads in both cases to the appearance of breaks in the kinetic curves for the inactivation of enzymes recorded in the low-temperature region, i.e. these curves are not described by a first-order equation over the entire time interval.[24-32] The positions of the breaks depend on the initial protein concentration in solution.[24-31]

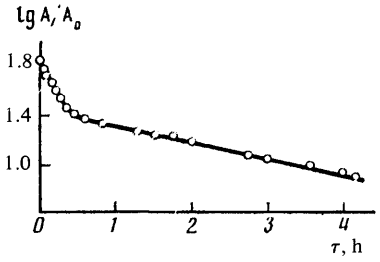


Figure 2. The semilogarithmic form of the kinetic curve for the inactivation of cytochrome P-450 in rabbit liver microsomes at 52 °C in tris-HCl buffer at pH 7.54.[50]

The complete kinetic characterisation of the protein inactivation process [scheme (2)] presupposes the determination of the rate constants for the forward (k_1 and k_2) and reverse (k_{-1}) reactions, the equilibrium constant between the native enzyme E and its denatured form A ($K = k_1/k_{-1}$), and also the expressions, in an integral form, for the concentrations of E and A at a specific instant. Analysis of scheme (2) subject to the initial conditions $t = 0$, $[E]_0 = a$, and $[A]_0 = [I]_0 = 0$ yields the following expressions for the current concentrations of E and A:[52]

$$E = a\left\{\frac{\lambda_2 - k_1^{-1}k_2}{\lambda_2(\lambda_2-\lambda_3)}e^{-\lambda_2 k_1 t} + \frac{k_1^{-1}k_2 - \lambda_3}{\lambda_3(\lambda_2-\lambda_3)}e^{-\lambda_3 k_1 t}\right\}$$

$$[A] = a/\lambda_2 - \lambda_3\cdot(e^{-k_1\lambda_3 t} - e^{k_1\lambda_2 t}), \text{ where } \lambda_2 = {}^1/_2(\alpha+\beta);$$

$$\lambda_3 = {}^1/_2(\alpha-\beta);\ \alpha = 1 + k_{-1}/k_1 + k_2/k_1;\ \beta = [\alpha^2 - 4k_2/k_1]^{1/2}$$

Evidently the current concentrations of the E and A forms of the protein are described by extremely complex equations. We may note that in practice it is frequently impossible to measure the concentrations of the protein forms A and I and one usually follows only the consumption of the initial enzyme E.

The system of equations describing scheme (2) and other similar schemes is non-linear and has no analytical solution.[30,32] In a series of studies[24-32] dealing in detail with the kinetics of the inactivation of oligomeric enzymes, Poltorak, Chukhrai, and co-workers therefore proposed several procedures for the approximate solution of the equations for the determination of the kinetic constants k_1, k_{-1}, and k_2 and the dissociation constants of the subunit enzymes K. One of the versions of the approximate solution makes it possible to obtain the rate constant for the dissociation of the oligomeric protein from the equation[31,32]

$$2(v/v_0) - {}^1/_2(v/v_0)^2 = {}^3/_2 - k_1 t\ , \qquad (4)$$

where v_0 and v are the rates of inactivation of the protein in the initial instant and after time t, which must not exceed the time τ corresponding to the break in the plot of ln v against t. In the second version of the approximate description of the kinetics of the inactivation of subunit enzymes, the following equation is used:[30-32]

$$(1/t)\ln v/v_0 = -k_1 + ({}^4/_3)k_1^2 k_{-1}[E]_0 t^2\ , \qquad (5)$$

in which $[E]_0$ is the initial concentration of the enzyme and the time t must also be less than τ. The linearisation of the kinetic inactivation curves with the aid of Eqn.(5) makes it possible to calculate not only the rate constant k_1 but also the rate constant k_{-1} for the reverse reaction and then also the constant for the dissociation of the subunit proteins into monomers or dimers in the thermal transformation of the tetrameric protein. Eqns.(4) and (5) have been used successfully to characterise kinetically the inactivation processes involving kidney acylase,[29] alkaline phosphatase,[31] and certain other oligomeric proteins.

Thus a correct kinetic description of the inactivation of enzymes with an arbitrary choice of the initial concentration reduces to a complex analysis of the kinetic curves, since their semilogarithmic forms have breaks, while the effective rate constants k_{in}, calculated with the aid of such semilogarithmic forms, are as a rule functions of the initial protein concentration and therefore strictly speaking are not constants. It is therefore necessary to seek methods for the approximate solution of the system of equations describing the associative-dissociative scheme (2) in order to find simple ways of linearing the kinetic relations.

Does this imply that there can be no cases where the kinetic curves for the thermal inactivation of enzymes can be linearised in terms of plots of ln v against t? In our view this is not so, since protein inactivation conditions such that the reversible dissociation of the subunit enzyme or the first reversible stage of the conformational transformation of the monomeric protein are reduced to a minimum or are altogether absent have been realised in practice.

First case. The subunit enzyme has been treated with bifunctional reagents and its reversible dissociation into monomers is difficult.[14,15,29,34] For example, kidney acylase modified by glutaraldehyde is inactivated in accordance with a first order law, since the stage involving the reversible dissociation of the enzyme has been virtually ruled out.[29] In this case, the semilogarithmic forms of the kinetic curves show no breaks of any kind when inactivation is continued up to extremely advanced stages.

Second case. The subunit enzyme dissociates into monomers via an "all or nothing" mechanism and the process is accompanied by the complete loss of catalytic function. As an example, one may quote the dissociation of tetrameric catalase into monomers in acid and alkaline media,[35] on treatment with denaturing agents,[53,54] in the course of lyophilisation,[55] and as a result of the introduction of succinyl groups into the protein by treatment with succinic anhydride.[36] Regardless of how the dissociation of catalase is initiated, this process is accompanied by the loss of catalase activity and its kinetics obey a first-order law even for high degrees of inactivation.[35,36,56]

Third case. The dissociation of the subunit protein into monomers is the rate-limiting stage of the entire inactivation process. In the study of an NAD-dependent hydrogenase, the validity of the dissociative scheme for the thermal inactivation of this tetrameric protein was demonstrated:[38]

$$E_{4\text{-in}} \overset{k_i}{\leftarrow} E_4 \overset{k_1}{\rightleftarrows} 2E_2 \overset{k_d}{\rightarrow} E_{2\text{-in}},$$

where k_i and k_d are the inactivation and denaturation rate

constants. The authors showed[38] that the rate of inactivation of the enzyme is determined by the dissociation of the tetramers into dimers. The semilogarithmic forms of the kinetic curves describing the loss of activity by hydrogenase therefore have no breaks up to considerable degrees of inactivation of the protein. In the case of monomeric proteins, there is also a possibility of inactivation pathways in which the rate of the entire process is limited by the first stage. Such examples will be presented below.

Fourth case. The protein is inactivated in highly dilute solutions where the association of the enzyme subunits or the aggregation of the monomeric protein is hindered or reduced to a minimum. In this case, subject to the condition that the rate-limiting stage of the entire process is the first reaction stage [see Scheme (2)], the inactivation kinetics obey a first order law and the semilogarithmic form of the kinetic curves should have no breaks of any kind.

We shall consider next the kinetics of the inactivation of proteins whose transformations under different conditions have been investigated in our laboratory.[35,36,47,50,56–70] They include monomeric (lysozyme,[47] myoglobin,[60] cytochrome b_5,[63,69] horseradish peroxidase,[61,62] and cytochrome P-450 LM-2[50,64,66–69]) and subunit biopolymers (lactate dehydrogenase,[58] malate dehydrogenase,[59] and catalase[35,36,56]). The thermal inactivation of all the proteins enumerated was studied

Table 1. Thermodynamic activation parameters for the irreversible inactivation of enzymes in different media.

Enzyme	Inactivation conditions	Δt[a]	E_{act}, kcal mol^{-1}	t° C	ΔH^*, kcal mol^{-1}	ΔS^*, cal mol^{-1} K^{-1}	ΔG^*, kcal mol^{-1}	Refs.
Glucose-6-phosphate dehydrogenase,	0.1 M NaOH-glycine buffer, pH 9.1	23—46	4.34; 131.40	25 45	3.75; 130.80	—70.20; 341.00	24.66; 22.40	[57]
EC 1.1.1.49	Inverted micelles; 0.1 M AOT[c] 0.1 M Triton X-45, 12% of 0.1 M NaOH-glycine buffer, pH 9.1	23—46	19.40; 61,42	25 45	18.80; 60.80	—18.15; 118.80	24.20; 23.02	[57]
Lysozyme, EC 3.2.1.17	0.067 M phosphate buffer, pH 6.2	20—80	24.20	37	23.60	—7.50	25.90	[47]
Lactate dehydrogenase, EC 1.1.1.27	0.1 M phosphate buffer, pH 8.2	20—60	6.40; 44.60	25 37	5.84 44.00	—60.06 63.50	23.70 24.40	[58]
Malate dehydrogenase, EC 1.1.1.37	0.1 M phosphate buffer, pH 8.2	20—52	43.00	37	42.40	63.60	22.70	[58,59]
Myoglobin from sperm whale heart	0.05 M phosphate buffer, pH 7.4 The same buffer with sodium cholate The same buffer with Twin 20 and 15% of glycerol	46—71 22—37 30—46	17.70 17.10 31.80	25 25 25	17.10 16.50 31.20	— —22.60 —24.00	— 23.20 24.00	[60] [60] [60]
Catalase, AC 1.11.1.6 Complex of catalase with strophanthin K	0.1 M phosphate buffer, pH 6.6 The same buffer	20—45 20—45	14.60 22.80	40 40	14.00 22.20	—30.20 —4.20	23.43 23.50	[56] [56]
Catalase subunits	0.1 M phosphate buffer, pH 3.28	22—40	14.70	37	14.10	—23.50	21.40	[35]
Succinylated catalase	0.1 M citrate-acetate buffer, pH 6.0	31—65	15.50	—	—	—	—	[36]
Horseradish peroxidase, EC 1.11.1.7	0.1 M citrate-acetate buffer, pH 6.0	37—53	19.90	45	19.20	—14.90	24.00	[61]
Complex of peroxidase with strophanthin K	The same buffer	37—53	20.60	45	19.90	—12.80	24.00	[61]
Horseradish peroxidase	0.05 M phosphate-citrate buffer, pH 7.0 Inverted micelles: 0.2 M AOT, 6% of 3 mM phosphate-citrate buffer, pH 4.6 Inverted micelles: 0.1 M AOT, 0.1 M Triton X-45, 6% of the same buffer, pH 4.0	45—80 26—50; 50—60 19—55; 55—66	47.40 26.40; 62.20 5.30; 62.40	45 45; 50.5 45	46.77 25.77; 61.56 4.67	64.71 6.33; 122.60 —61.42	26.19 23.76; 21.90 24.20	[62] [57,62] [57,62]
Cytochrome b_5 from rabbit liver	0.05 M tris-H_3PO_4 buffer, pH 7.5 with 15% of glycerol Inverted micelles: 0.25 M AOT, 4.5% of the same buffer Inverted micelles: 0.13 M AOT, 0.13 M Triton X-45, 4.5% of the same buffer	50—62 32—39 31—37	4.80 2.00 6.60	50.5 37 37	4.16 1.39 6.00	—63.84 —71.75 —54.74	24.80 23.63 23.00	[63] [63] [63]
Cytochrome P-450 LM-2, EC 1.14.14.1	0.05 M Tris-HCl buffer, pH 7.54, with 20% of glycerol	40—58	23.50	45	22.86	—7,25	25.17	[50]
Cytochrome P-450 immobilised on polyamide	The same buffer	40—58	22.40	45	21,76	—8.47	24.46	[50]
Cytochrome P-450 in microsomes	0.05 M phosphate buffer, pH 7.33, with 0-4.1 M glycerol 0.05 M tris-HCl buffer, pH 6.88, with 0-4.1 M glycerol	45—54 45—54	111.0—54.0 110.0—98.0	50.4 50.5	110.0—53.0 109.0—97.0	280 0—89 0 265.0—220.0	19.70—24.80 23.80—26.20	[64,65] [64,65]

[a] Δt = temperature range.
[b] The temperatures for which the thermodynamic parameters were calculated.
[c] AOT = Aerosol OT (sodium salt of di-2-ethylhexyl ester of sulphosuccinic acid).
Pairs of activation parameters are quoted in those cases where the temperature variations of the constants exhibit a break in the Arrhenius plots.

in buffer solutions and, for some of them (peroxidases,[57,62] glucose-6-phosphate dehydrogenase,[57] cytochrome b_5,[68,69] and cytochrome P-450 LM-2[66–69]), in inverted surfactant micelles in organic solvents. The thermal inactivation of the integral membrane enzyme cytochrome P-450 LM-2 was studied in biomembranes (liver microsomes).[50,64,65] Among the test objects, there are water-soluble (lysozyme, peroxidase, catalase, myoglobin, and dehydrogenases) and hydrophobic biopolymers (cytochromes b_5 and P-450), as well as modified enzymes (succinylated catalyse,[36] systems obtained by coupling strophanthin K to peroxidase,[61] and catalase[56]). The choice of these test objects was dictated not only by fundamental research but also the wide-scale employment of the majority of them in practice.[71–73] The proteins which we investigated, the conditions and temperatures ranges in which they are inactivated, and the thermodynamic activation parameters are listed in Table 1.

We confined ourselves to a quantitative examination of data obtained solely in our laboratory, although Table 1 might have been supplemented by the results of studies of other groups in our country[14,15,19,20–34,74,75] and abroad.[17,76–80] This limitation of the range of kinetic studies of the thermal inactivation of proteins (enzymes) considered is due to the following causes: firstly, the data of other workers have already been surveyed and examined critically in earlier publications[14,15,19–22] or in the course of the preparation of the present review[74,75] and in our view does not require a fresh analysis; secondly, the quantitative characteristics of thermal inactivation have been frequently obtained by different workers under different conditions, so that it is sometimes difficult to establish the initial concentrations of the proteins subjected to thermal inactivation, which leads to contradictory results when the effective kinetic constants are referred to particular stages of the protein inactivation and reactivation processes investigated, which have been analysed, for example, in a recent monograph.[81] Analysis of the kinetic constants for the thermal inactivation of proteins without referring them to the initial concentrations of the inactivated objects is incorrect, as will be shown below, and can lead to erroneous conclusions about the character and mechanism of the thermal inactivation of biopolymers. We have therefore confined ourselves to a quantitative consideration of our own data, since the results analysed here have been obtained under controlled conditions, which can be readily compared for different objects in different media.

III. KINETICS OF THE INACTIVATION OF ENZYMES IN DIFFERENT MEDIA

1. Kinetics of the Inactivation of Enzymes in Aqueous Media

Lysozyme (EC 3.2.1.17) is inactivated in accordance with scheme (2).[41,44–47] At protein concentrations in the range 0.14–5.48 M (in the temperature range 40–70 °C), the first order rate constant depends on the initial concentration (Fig.3), the dependence being described by the equation

$$k_{in} = k_0 \exp(-\alpha[E]_0), \quad (6)$$

where $[E]_0$ is the initial protein concentration, k_0 the inactivation rate constant at infinite dilution of the protein which is independent of $[E]_0$, and α a coefficient which depends on temperature and has the dimensions of the reciprocal of concentration.[47] The temperature variation of α (Fig.3) is described by the equation

$$\alpha = \alpha_0(1 - \gamma/T) \quad (7)$$

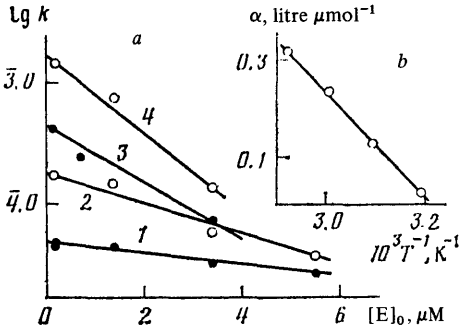


Figure 3. *a*) Dependence of the effective rate constants for the inactivation of lysozyme on its initial concentration at different temperatures (°C): *1*) 40; *2*) 50; *3*) 60; *4*) 70; *b*) temperature dependence of α.[47]

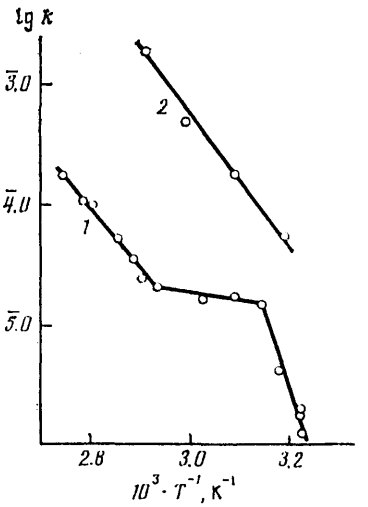


Figure 4. Temperature dependence of the rate constants for the inactivation of lysozyme at a concentration of 0.20 mg ml^{-1} (curve *1*) and at infinite dilution of the enzyme (curve *2*).[47]

The temperature variation of the effective inactivation rate constants k_{in} at high lysozyme concentrations is characterised by breaks in the Arrhenius plot (Fig.4), while the temperature variation of the rate constants k_0, obtained by extrapolating k_{in} to zero protein concentration, does not exhibit breaks (Fig.4, line *2*). E_{act} and the expression for the rate constants in a general form can be found from Fig.4 (line *2*):[47]

$$k_0 = 4.0 \times 10^{11} \exp(-24\,200/RT). \quad (8$$

From Eqns.(6)–(8), we obtain a general expression for the

effective rate constant k_{in}:[47]

$$k_{in} = 4.0 \times 10^{11} \exp\{-24\,200/RT - \alpha_0(1-\gamma)T[E]_0\} \quad (9)$$

Evidently, the temperature dependence of k_{in} is not described by the Arrhenius law, since k_{in} depends not only on temperature but also on the protein concentration $[E]_0$. The correct kinetic constant characterising the inactivation of lysozyme can only be k_0, since it does not depend on the protein concentration in solution.

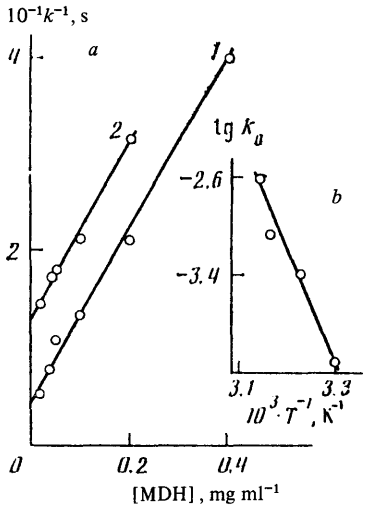


Figure 5. *a*) Dependence of the effective rate constants for the thermal inactivation of malate dehydrogenase (MDH) on the initial enzyme concentration at 45 °C (curve *1*) and 43 °C (curve *2*); *b*) temperature dependence of the rate constant k_0 for the inactivation of malate dehydrogenase corresponding to the infinite dilution of the protein.[59]

Malate dehydrogenase (EC 1.1.1.37) is inactivated in accordance with a scheme analogous to scheme (2):

$$E_2 \overset{k_1}{\rightleftarrows} 2E \overset{k_d}{\rightarrow} E_d,$$

in which E_2 is the dimer, E the enzyme subunit, k_1 the rate constant for the dissociation of the dimer, and k_d the rate constant for the denaturation of the subunit.[59] For an arbitrarily selected protein concentration, the inactivation rate constants k_{in}, evaluated from the semilogarithmic forms of the kinetic curves describing the decrease of the activity of the enzyme, depend on the initial dimer concentration (Fig.5):[59]

$$k_{in} = k_0/(1+\alpha k_0[E_2]_0) \quad (10)$$

where k_0 is the rate constant for the inactivation of the protein at infinite dilution and α is a constant which depends on temperature and has the dimensions of the reciprocal of the rate (litre mol^{-1} s). The temperature variation of the rate constant for the inactivation of malate dehydrogenase k_0 (Fig.5) is described by the equation:[59]

$$k_0 = 1.42 \times 10^{27} \exp(-43\,000/RT) \quad (11)$$

The temperature variation of the effective rate constants k_{in} is not described by the Arrhenius equation, since they depend on temperature, the enzyme concentration, and the coefficient α.

Table 2. The rate constants k_{in} for the inactivation of malate dehydrogenase (MDH), calculated from the lg v–time relations,[59] and the rate constants k_1 calculated from Eqn.(4).[31,32]

$[MDH]_0 = 0.01$ mg ml^{-1}			$[MDH]_0 = 0.05$ mg ml^{-1}		
t °C	$10^4 k_{in}$, s^{-1}	$10^4 k_1$, s^{-1}	t °C	$10^5 k_{in}$, s^{-1}	$10^5 k_1$, s^{-1}
20	0.128	0.139	28	0.798	1.670
25	0.294	0.278	30	3.220	2.900
26.5	0.319	0.296	34	10.01	9.52
28	0.437	0.389	37	11.70	12.30
30	0.511	0.407	38	11.80	12.50
34	0.766	0.714	40	19.30	17.20
37	1.440	1.480	43.5	69.00	62.50
40	2.920	3.180	46	115.00	105.00
45	9 590	9.170	49	478.00	400.00

It is of interest to compare the effective rate constants k_{in}, calculated for different initial protein concentrations and temperatures, with the rate constants k_1 (see the inactivation scheme presented above) calculated from Eqn.(4).[31,32] Table 2 compares the values of k_{in} and k_1 under different conditions. At low enzyme concentrations, the two constants differ little, but, with increase of the concentration of malate dehydrogenase by a factor of 5, the difference between them becomes significant. We believe that the agreement between k_{in} and k_1 at low protein concentrations is not fortuitous. The stage involving the dissociation of the enzyme dimers is rate limiting in its inactivation and the reverse stage, involving the reassociation of the malate dehydrogenase subunits at low enzyme concentrations, is difficult or does not occur at all. Nor can one rule out the possibility that the inactivation is limited by the conformational changes in the dimer, occurring when it dissociates into subunits.

Lactate dehydrogenase (EC 1.1.1.27). It is a tetrameric enzyme, whose inactivation can be described by several schemes with successive dissociation into dimers and monomers.[58] In the temperature range 20–60 °C, the effective inactivation rate constant depends on the initial concentration of the tetramer E_4. This dependence is described by Eqn.(10), in which the concentration of the dimer E_2 has been replaced by the concentration of the tetramer E_4. In the range 35–60 °C, the temperature dependence of k_0 obeys the equation:[58]

$$k_0 = 1.30 \times 10^{27} \exp(-44\,600/RT) \quad (12)$$

Below 35 °C, E_{act} falls to 6.40 kcal mol^{-1}.

Catalase (EC 1.11.1.6) from ox liver. The dissociation of the enzyme obeys the "all or nothing" principle: $E_4 \overset{k_1}{\rightleftarrows} 4E$.[53,54] The enzyme subunits lack catalase activity.[35,36,53,55] The constant for the dissociation of catalase into subunits k_1 depends strongly on the pH and is described by Eqns.(13) and (14) respectively in acid and alkaline media:[35]

$$k_1 = 5.0 \times 10^7 [H^+]^{2,3}, \quad (13)$$

$$k_1 = 5.0 [H^+]^{-1,4} \quad (14)$$

In 0.1 M phosphate buffer at pH 6.6 at temperatures in the range 20–45 °C, the thermal inactivation of catalase (investigated in terms of the loss of its catalytic activity in the oxidation of ethanol by cumenyl hydroperoxide) is characterised by E_{act} = 14.6 kcal mol^{-1}. After its modification by 11 molecules of strophanthin K, the activation energy for the thermal degradation of the enzyme increases to 22.8 kcal mol^{-1}.[56] The temperature variation of the rate constant for the inactivation of catalase in the range 20–45 °C is described by the equation[56]

$$k_1 = 4.13 \times 10^6 \exp(-14\,600/RT) .$$

Horseradish peroxidase (EC 1.11.1.7). The temperature variation of k_{in} for the enzyme is described by the equation[61]

$$k_{in} = 1.7 \times 10^{10} \exp(-19\,900/RT) .$$

The modification of peroxidase by three strophanthin K molecules alters little the activation characteristics for the inactivation of the enzyme (Table 1). A change in the pH has a marked influence on the rate constant for the inactivation of peroxidase and the activation characteristics of the process: in 0.1 M citrate-acetate buffer at pH 6.0, the activation energy is 19.9 kcal mol^{-1},[61] while in 0.05 M phosphate-citrate buffer pH 7.0 it increases to 47.4 kcal mol^{-1}.[62]

Cytochrome P-450 LM-2 (EC 1.14.14.1). In tris-HCl buffer (pH 7.54) with 20% of glycerol, the enzyme loses its catalytic activity in the range 40–58 °C. E_{act} = 23.5 kcal mol^{-1} corresponds to this process.[50] It is important to note that the loss of catalytic activity is accompanied by the simultaneous decrease of the intensity of the Soret band of the carbonyl complex of cytochrome P-450 and by the appearance of a spectral band at 420 nm, corresponding to the inactive form of the haemoprotein, namely P-420.[65,82] The immobilisation of cytochrome P-450 LM-2 on polyamide does not induce an appreciable change in E_{act} for the thermal degradation of the haemoprotein (22.4 kcal mol^{-1}).[50] The kinetic curves for the decrease of the catalytic activity of cytochrome P-450 LM-2 in solution as a rule correspond to several stages which can be accounted for in two ways: firstly, the highly hydrophobic cytochrome P-450 forms aggregates in aqueous solution in which it is inactivated at different rates; secondly, in dilute solutions the inactivation of different conformers of the same form of cytochrome P-450 may be manifested, since after extraction from microsomal membranes the haemoprotein is unusually labile in aqueous media.

2. Kinetics of the Inactivation of Enzymes in Inverted Surfactant Micelles in Organic Solvents

Inverted surfactant micelles, in which enzymes and proteins are incorporated, constitute a realistic model of biological membranes, reflecting many important aspects of their structure and complex lipid–protein and protein–protein interactions.[66–69,83–85] Furthermore, microheterogeneous (protein–surfactant–water–organic solvent) systems constitute an extremely interesting medium for enzymic reactions.[83–85] It has been frequently demonstrated that enzymes of different classes, solubilised by inverted surfactant micelles, not only retain their catalytic properties but are sometimes more active than in aqueous media.[69,83–86] For the practical use of proteins in inverted surfactant micelles, it is important to know not only the catalytic activity of the biocatalysts under these conditions but also their stability at different temperatures and pH and on exposure to the influence of denaturing additives. We have shown that the stability of haemoproteins in inverted surfactant micelles depends on temperature, pH, ionic strength, and protein stabilising additives, as in aqueous media; the stability of haemoproteins is also influenced by the physicochemical properties and composition of the micelles, by the specific alteration of which it is possible to stabilise significantly the solubilised proteins.[66–69,83–85]

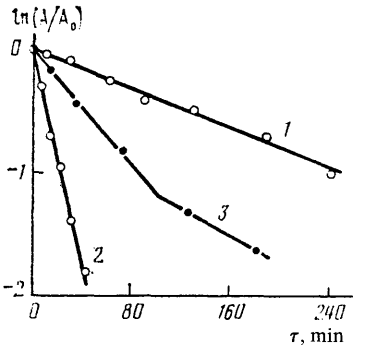


Figure 6. Semilogarithmic forms of the kinetic curves for the inactivation of glucose-6-phosphate dehydrogenase at 40 °C: *1*) buffer solution; *2*) AOT micelles in heptane; *3*) mixed AOT–Triton X-45 micelles in heptane;[57] the conditions are indicated in Table 1.

Glucose-6-phosphate dehydrogenase (EC 1.1.1.49, G6PDH) is inactivated in inverted AOT micelles (AOT = sodium salt of di-2-ethylhexyl ester of sulphosuccinic acid) in heptane in accordance with a first-order kinetic law up to high degrees of inactivation (Fig.6).[57] We may note that the enzyme is inactivated in aqueous solutions also in accordance with the same law. However, in mixed micelles made up of AOT and Triton X-45 (1:1), the inactivation of the enzyme deviates from the first-order law (Fig.6). The temperature variation of the effective rate constants for the inactivation of G6PDH in the range 23–46 °C is characterised by a break in the Arrhenius plot at 34 °C. The activation energy is E_{act} = 61.4 kcal mol^{-1} above the break and 19.4 kcal mol^{-1} below it.[57] On inactivation of the enzyme in aqueous solutions, a break is also observed at 36 °C and E_{act} differs from the values calculated for micellar systems (Table 1).

Horseradish peroxidase exhibits a "periodicity" in the variation of its activity on inactivation in inverted AOT micelles and in mixed micelles comprising AOT and Triton X-45, which we attribute to complex cooperative transformations of the "water–protein–AOT–heptane" system.[57,62] This "periodicity" in the inactivation of the peroxidase is smoothed out on raising the temperature to 50–55 °C (Fig.7). We may note that in buffer solutions the inactivation of peroxidase is described by a first-order kinetic equation (Fig.7*b*, lines 5 and 6),[57,61,62] i.e. the phenomenon of "periodicity" in the variation of the catalytic activity of the enzyme is associated solely with the nature of the inverted surfactant micelles. The rate constants for the inactivation of the peroxidase in micelles are also influenced by the concentration of the buffer, its pH, the percentage content of the aqueous phase, and the addition of the non-ionic detergent Triton

X-45 or glycerol to the system.[57,62] The temperature variation of the effective rate constants for the inactivation of the peroxidase in micelles is characterised by a break at 55 °C in the Arrhenius plot, and the values of E_{act} above and below this temperature differ markedly (Table 1).

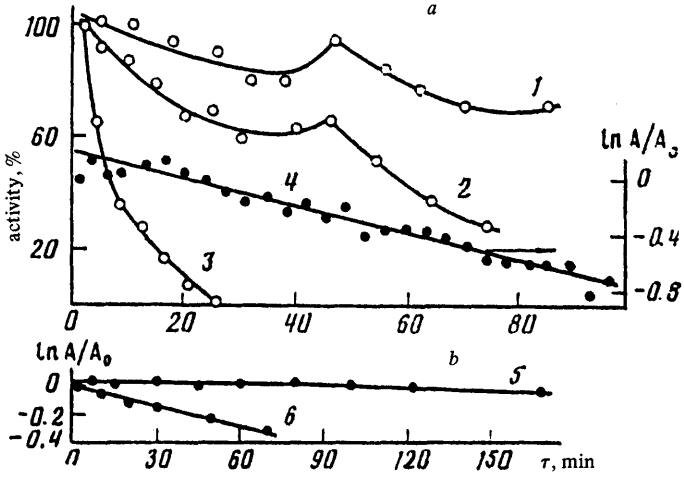


Figure 7. Kinetic curves for the variation of the catalytic activity of peroxidase in inverted micelles; 0.2 M AOT, 6% of polar phase comprising 0.05 M phosphate-citrate buffer at pH 4.6 (13.5 nM enzyme): *a*) temperature (°C) [*1*) 37; *2*) 43; *3*) 54; *4*) semilogarithmic form of the kinetic curve at 40 °C]; *b*) semilogarithmic forms of the kinetic curves for the variation of the activity of peroxidase (13.5 nM) in 0.05 M phosphate-citrate buffer at pH 7.0 at different temperatures (°C) [*5*) 45; *6*) 55].[57]

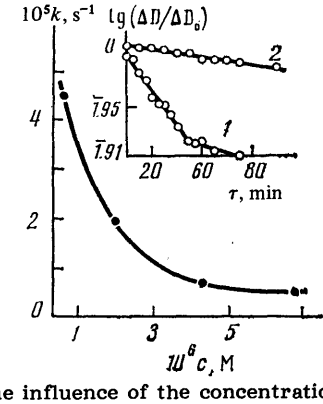


Figure 8. The influence of the concentration of cytochrome b_5 (*c*) on the rate constant for its transformation in AOT micelles in octane at 31 °C; cytochrome b_5 concentration (μM): *1*) 0.68; *2*) 6.84; (0.25 M AOT, 45% of polar phase in octane).[63]

Cytochrome b_5 is inactivated in inverted micelles at rates which depend on its initial concentration (Fig.8). At low protein concentrations, the breaks in the semilogarithmic forms of the kinetic curves describing the decrease of the intensity of the Soret band are absent but they appear on raising the concentration of the haemoprotein.[63] The character of the variation of k_{in} with the initial concentration is the same for both cytochrome b_5 in micellar systems and for malate and lactate dehydrogenases in aqueous solutions [Eqn.(10)]:[59] with increase of the concentration of the protein, the rate constant for its inactivation diminishes.

Cytochrome P-450 LM-2 is inactivated at different rates in micelles of different composition.[66-69] By increasing the complexity of the micelles, it is possible to achieve a significant stabilisation of this labile protein in micellar systems. At 40 °C, k_{in} for cytochrome P-450 in micelles containing AOT, cetyltrimethylammonium bromide, Twin 20, and Triton X-45 is 2.9×10^{-6} s^{-1}, while in liver microsomes at the same temperature it is 3.4×10^{-6} s^{-1},[66,67] i.e. the stability of the haemoprotein in a specifically selected micellar system can be actually greater than its stability in the native membrane.[66,67,69]

Thus the inverted surfactant micelles not only protect proteins against the denaturing influence of organic solvents but in certain cases are capable of ensuring their relatively high thermal stability in a hydrocarbon medium.[66-69] The optimum composition of the inverted micelles ensuring the retention of the catalytic activity of the enzyme or even its increase is specific to each protein. However, the thermal stability of enzymes in micelles is usually lower than in an aqueous medium, although enzymes in micellar systems can be stabilised by increasing the complexity of the composition of the micelles in such a way that the stability of the enzyme reaches a level characteristic of buffer solutions. For example, at 45 °C the rate constant for the inactivation of G6PDH in a buffer solution is 2.24×10^{-3} s^{-1}, while in mixed 1:1 micelles comprising AOT and Triton X-45 it is 9.12×10^{-4} s^{-1}, i.e. the enzyme is 2.4 times more stable in the micelles.[57] Yet another way of stabilising proteins in organic solvents has been proposed recently:[87] by the covalent incorporation of α-chymotrypsin in particles of cross-linked polyacrylamide gel with colloidal dimensions (100–400 Å). This catalyst is universal and can be used in both hydrocarbon and aqueous solutions.

The quantitative description of the inactivation of proteins and micelles is associated with the same difficulties as in aqueous solutions: the effective inactivation rate constants depend on the initial protein concentration;[63,70] the temperatrue variation of these constants is complicated by breaks in the Arrhenius plots, which reflect not only the effective character of the rate constants but also the characteristic features of the behaviour of the micellar systems as the temperature is raised.[57,62,70]

3. Kinetics of the Inactivation of Cytochrome P-450 in Microsomal Membranes

Cytochrome P-450 is the terminal oxidase in many hydroxylating multienzyme systems, widely distributed in nature.[82] These systems are responsible for the hydroxylation of hydrophobic compounds to form more polar hydroxy-derivatives, which are either removed from the organism or serve as key compounds in the biosynthesis of bioactive substances.[82] The majority of cytochromes P-450 are associated to different extents with membranes and are distinguished by a high lability after extraction from the native environment. The unique ability of cytochrome P-450 to catalyse the conversion of a wide variety of compounds into hydroxy-derivatives has led investigators to seek ways of the practical utilisation of

this family of biocatalysts. However, the difficulty of isolating the cytochromes from the membrane, their low stability in aqueous media, the high cost of the source of the raw material, and also the multienzyme nature of the hydroxylating systems have made it necessary to seek procedures whereby stabilised organelles (microsomes, mitochondria) could be used in enzymic hydroxylation processes *in vitro*.[88,89] It is therefore a matter of great urgency to be able to characterise the inactivation of cytochromes P-450 in native membranes and to stabilise them in this medium.[50,64,65,82,88,89] We investigated the inactivation of cytochrome P-450 in rabbit liver microsomes on the basis of the loss of its catalytic activity in the oxidation of aniline and the variation of its spectroscopic properties.[50,64,65]

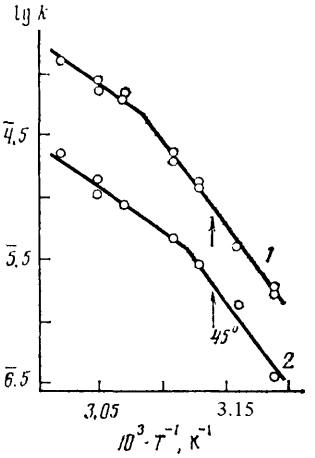


Figure 9. Temperature dependence of the rate constants for the thermal inactivation of cytochrome P-450 in rabbit liver microsomes in the absence of glycerol from the solution: *1*) first stage of the process; *2*) second stage of the process.[50]

The decrease of the catalytic activity of the haemoprotein in microsomes is accompanied by a decrease of the intensity of the Soret band of its CO complex and an increase of the concentration of the inactive form of the haemoprotein—cytochrome P-420.[65] The change in the activity of cytochrome P-450 in microsomes takes place in several stages.[64] As a rule, the effective rate constant has been determined solely for the first stage of the process. The temperature variation of k_{in} for cytochrome P-450 in microsomes is characterised by a break in the Arrhenius plots for the first and second stages of the haemoprotein inactivation process (Fig. 9).[50] The causes of the appearance of the breaks will be discussed below. One of the ways of stabilising membrane proteins, and cytochrome P-450 in particular, is by incorporation in lipid dispersions and liposomes, modelling many aspects of native biological membranes. This problem is discussed in detail in a monograph.[90] The study of the thermodynamic characteristics of the thermal inactivation of cytochrome P-450 in liposomes showed that they depend on the pH and temperature and vary, compared with aqueous solutions, in the following ranges: ΔH from 78 to 80 kcal mol^{-1} and ΔS from −163 to −184 cal mol^{-1} K^{-1}. This indicates an increase of the conformational "rigidity" of the protein in liposomes compared with buffer solutions.[91]

An important task in the study of the thermal inactivation of enzymes is the correlation of the kinetic characteristics of the rate-limiting stage of this complex process and its molecular mechanism. The solution of this problem by kinetic procedures alone is impossible and requires the use of many other methods (electronic, circular dichroism, and NMR spectroscopy, sedimentation analysis, and electrophoresis). The application of a set of physicochemical methods makes it possible in many instances to relate the rate-limiting stage of the thermal inactivation to fairly definite molecular mechanisms of the transformations of the biopolymer. It has been already noted that ^{13}NMR,[43] fluorescence,[45] and circular dichroism[46] data, measurement of the optical density of lysozyme,[42] and the study of the kinetics of its inactivation[44,47] suggest that the rate-limiting stage in the thermal inactivation of this protein is associated with the ionisation of the Glu-35 residue and the decomposition of the polypeptide chain along the 5–15 section.[47–49] The rate-limiting stage in the thermal inactivation of the more complex membrane enzyme cytochrome P-450 is the cleavage of the coordinate bond between the haem iron and the cysteine residue in the polypeptide chain, which transforms the enzyme into the inactive form P-420.[50,82,90] The determination of this rate-limiting stage became possible solely from a series of studies of the kinetics of the thermal inactivation of cytochrome P-450[50,65] and of the changes in its spectroscopic characteristics.[65,82,90]

Studies of Poltorak, Chukhrai and co-workers,[24–33,74] Berezin, Martinek, and Ugarova and co-workers[14,15,19–22,75] in our country and of Pfeil[17] and Jaenicke[76–80] abroad have been devoted to the solution of the complex problem of correlating the kinetic parameters and the structural changes in enzymes during their inactivation. Examination of these problems is very important but is outside the framework of this review, which is devoted solely to the kinetic aspects of the thermal inactivation of proteins and enzymes. We can only refer the reader to a recent review,[74] which deals in detail with the processes involving the dissociative thermal inactivation of enzymes and the approximate methods of calculation of the individual kinetic parameters of these processes and indicates ways whereby subunit enzymes can be stabilised by the fixation of their quaternary structure, and also to a review[75] devoted to the principal structural and physicochemical factors governing the stabilisation of protein macromolecules in solution and methods for the stabilisation of biocatalysts by chemical modification and point mutations of proteins. A useful critical analysis of studies concerning the correlation of the kinetic data for the inactivation and reactivation of subunit enzymes and their structural changes has been carried out in a monograph.[81]

IV. THE INFLUENCE OF POLYOLS AND DETERGENTS ON THE STABILITY OF ENZYMES IN SOLUTIONS

The medium in which proteins function in the living cell differs to a large extent in composition, viscosity, polarity, and other parameters from the solutions used *in vitro*. It has long been known that various organic solvents greatly reduce the stability of proteins. The addition of many low-molecular-weight compounds (salts, sugars, polyols, detergents, etc.) to protein solutions influences the stability of the proteins (enzymes), since such substances alter the properties of the solutions and the affinity of the protein for the solvent. The influence of certain low-molecular-weight additives on the thermal stability of proteins is analysed in this Section and the mechanism of the stabilisation of the proteins is discussed.

The stabilising effect of polyols on proteins is well known.[92-102] The degree of denaturation of ovalbumin by urea decreases in the presence of sucrose,[92] polyhydric alcohols increase the temperature of the thermal transition of lysozyme and ribonuclease,[93] and sucrose has a stabilising effect on egg proteins.[93] Various methods have been used to investigate the quantitative characteristics of the stabilising action of polyols on proteins:[98,100,101] systematic studies have been made of the influence of a series of polyols on the denaturation temperatures of ovalbumin, lysozyme, conalbumin, and α-chymotrypsinogen by the calorimetric method[98] and the interaction of a number of proteins with aqueous solutions of lactose and glucose has been studied in detail by the densitometric method.[100] In our laboratory a kinetic approach has been used to characterise quantitatively the influence of polyols on the thermal stability of various proteins.[58,61,64,65] The stabilising influence of polyols has been studied in relation to horseradish peroxidase modified with three strophanthin K molecules,[61] lactate dehydrogenase in aqueous solutions,[58] and cytochrome P-450 in rabbit liver microsomes.[64,65] Regardless of the nature of the protein (dissolved in water or hydrophobic) and its structural organisation (monomeric or subunit) and also regardless of the environment of the enzyme (aqueous solution or microsomal membranes), all the low-molecular-weight polyols and dextrans decrease the rate of inactivation of proteins.[58,61,64,65]

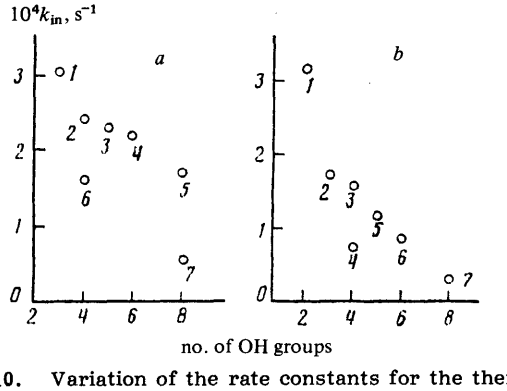


Figure 10. Variation of the rate constants for the thermal inactivation of horseradish peroxidase modified with strophanthin K (*a*) and of cytochrome P-450 in microsomes (*b*) with the number of OH groups in the polyol molecule; *a*: *1*) glycerol; *2*) rhamnose; *3*) glucose; *4*) sorbitol; *5*) maltose; *6*) xylose; *7*) sucrose; 50 °C;[61] *b*: *1*) ethylene glycol; *2*) glycerol; *3*) rhamnose; *4*) xylose; *5*) glucose; *6*) sorbitol; *7*) maltose; 50.5 °C.[64]

An increase of the concentration of glycerol and sugars in solution is accompanied by a monotonic decrease of the rate of inactivation of the peroxidase–strophanthin complex,[61] lactate dehydrogenase,[58] and cytochrome P-450 in microsomes.[64,65] Poly(ethylene glycols) 600, 1000, and 6000 and dextrans 20, 40, and 500 also stabilise the peroxidase–strophanthin complex.[61] However, dextran 20 and poly(ethylene glycols) 300 and 1000 have no stabilising effect on cytochrome P-450 in microsomes.[64] The question arises how the data concerning the stabilising action of polyols can be explained.

We showed that there is a direct relationship between the rate constants for the inactivation of the peroxidase complex and cytochrome P-450 and the number of OH groups in the molecules of stabilising additives (Fig.10): the greater the number of OH groups present in the stabiliser, the lower the value of k_{in}, i.e. the more stable the protein in solution or in microsomes.[61,64,65] In order to account for these data, we may recall the principal factors governing the stabilisation of proteins.[14,15,98] The stability of proteins is promoted by hydrogen bonds, electrostatic and hydrophobic interactions, and complex formation with certain metals. It is known that the dissolution of polyols in water has little effect on the formation of hydrogen bonds by proteins.[98] Electrostatic interactions are usually stronger in solutions of sugar than in water, but their contribution to the stabilisation of proteins is relatively small. The main factor stabilising the steric structure of proteins is hydrophobic interactions.[1-3,14,15,98] Such interactions are determined to a large extent by the nature of the solvent and hence depend greatly on the way that sugars and other polyols influence the structure of water. It has been shown that the interaction between pairs of hydrophobic groups is much stronger in solutions of glycerol and sugars than in pure water.[98] This is probably the main mechanism by means of which polyols stabilise proteins in aqueous solutions. The stabilising action of polyols is determined by the extent of their stabilising effect on aqueous solutions. The relative quantity $f = (\bar{V}_2 - V_w)/V_w$, where V_w is the van der Waals volume of the solute and $\bar{V}_2$ the partial molar volume of the same substance, including the internal volume of the molecule and the changes in the latter induced by the action of the solvent, has been suggested[98] as a structure formation characteristic of this kind. As an example we may quote of f for glycerol in water at 25 °C:[103] V_w = 51.4 ml mol^{-1}, $\bar{V}_2$ = 73.5 ml mol^{-1}, and f = 43.1%. The van der Waals volumes of sugars and other polyols have been calculated.[104] The following equation is used to determine the partial molar volumes $\bar{V}_2$:

$$\varphi_v = M_r/d_0 = 1000(d - d_0)/cd_0 ,$$

where d and d_0 are the densities of the solution and the solvent, M_r is the molecular weight of the solute, and c the molar concentration of the solute in the solution.[98] Evidently ϕ_V varies linearly with c and extrapolation to zero concentration can yield the partial molar volume $\bar{V}_2$. The partial molar volumes of many sugars have been calculated in this way and have been published.[105] Sugars induce effective structure formation in water when their OH groups are oriented in space in such a way that the distance between them corresponds to the distance between the oxygen atoms in the molecular lattice of water.[98] More accurate quantitative characteristics of structure formation in water by polyols are not at present available.

There exist also other explanations of the stabilising effect of polyols on proteins. It has been suggested that polyol molecules can penetrate into the protein globules, which tends to intensify the hydrophobic interactions within the globule and to lower its lability.[99] In certain cases there is a possibility of specific binding of sugars and alcohols by proteins. For example, in the crystalline state lysozyme interacts with glucose, which stabilises the enzyme, since it is bound to it at at least two centres.[106] The constant for the binding of glucose to lysozyme is 2.3 M^{-1}.[106]

The stabilising influence of polyols on proteins of a wide variety of kinds indicates the principal role of the structure of the aqueous medium in which proteins are present and the secondary role of the structural organisation of the proteins themselves. It is of interest to analyse the influence

of polyols on cytochrome P-450 in the microsomal membrane, i.e. in a microenvironment different to that in its aqueous solution.

Low-molecular-weight and high-molecular-weight polyols act on microsomes in different ways. The low-molecular-weight additives influence the membrane as mild surfactants: with increase of temperature, they penetrate into the lipid double layer, increase the degree of the hydration of phospholipids, lower the surface viscosity of the double layer, and increase the lateral mobility of the phospholipids.[107-110] High-molecular-weight additives poly(ethylene glycols) and dextran do not penetrate the lipid double layer, but they do reduce the surface potential of phospholipids and lead to the aggregation of vesicles;[111] by competing with phospholipids for water, they cause the contraction of the liquid crystal lattice of the double layer.[112] Thus low-molecular-weight polyols can act on the membrane as a whole, while high-molecular-weight polyols influence significantly only its surface layer. This can explain the different effect of polyols on the microsomal cytochrome P-450.[64,65] The stabilising effect of polyols must therefore be characterised taking into account their influence on the hydrophobic interactions in the protein globule and throughout the membrane as a whole. By penetrating into microsomes, glycerol promotes hydrophobic contact between pairs of non-polar groups of the cytochrome P-450 molecule, while in the regions near the membrane the polarity of the medium is reduced. Glycerol equalises the properties of the entire system and greatly increases the degree of structure formation in the medium. The considerable decrease of the entropy of activation with increasing glycerol concentration in the inactivation of microsomal cytochrome P-450 can be understood from this standpoint. On the other hand, glycerol decreases the enthalpy of activation for the thermal denaturation of cytochrome P-450 by acting on microsomes as a detergent and significantly facilitates the mobility and lability of the haemoprotein in the lipid double layer.[64,65] The influence of glycerol on the enthalpy of activation can be regarded as a destabilising factor in the thermal inactivation of cytochrome P-450, while the influence of glycerol on the entropy of activation is a stabilising factor in the thermal inactivation of the haemoprotein. It follows from our data that the ratio of these two factors favours the entropic factor, as a result of which the free energy of activation for the degradation of cytochrome P-450 in the presence of glycerol increases and the rate constant for the thermal degradation of cytochrome P-450 therefore diminishes. We may note that similar ratios of ΔH^* and ΔS^* have also been observed for other methods of stabilishing proteins, for example, when they are immobilised on carriers:[23] the binding to the matrix results in a deviation from the initial structure of the enzyme towards its denaturation, in a decrease of ΔH^*, and in a simultaneous decrease of ΔS^* as a consequence of the ordering of the structure of the immobilised protein.

Compared with polyols, detergents have a greater influence on proteins in aqueous solution. Systematic studies of the interaction of detergents with proteins in aqueous solutions are essential in view of their practical importance. Firstly, the use of detergents is inevitable in the isolation from membranes of the proteins bounds to them.[82,113-115] Secondly, the solubilisation of water-insoluble substrates of lipolytic enzyme is frequently necessary.[116] Thirdly, in order to attain high rates of enzymic reactions, non-ionic detergents are frequently added to buffer solutions.[117,118] In most cases the detergents are selected empirically. Extensive experimental data on the interaction of detergents with soluble and membrane proteins have been surveyed in a number of reviews.[115,119,120] However, the absence of systematic studies of the interaction of various detergents with proteins is still felt as an acute problem, which hinders the survey and assessment of the prospects for the development of this methodologically important field of enzymology.

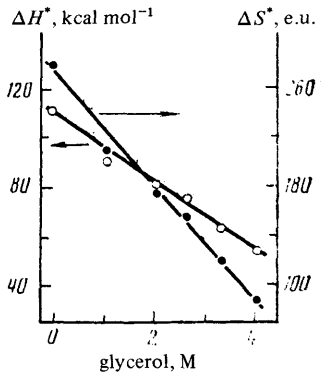


Figure 11. Dependence of ΔH^* and ΔS^* on the glycerol concentration in the inactivation of cytochrome P-450 in microsomes in a phosphate buffer at pH 7.3.[64]

Systematic studies of the interactions of various detergents in an aqueous medium with myoglobin and cytochromes c and P-450 have been carried out in our laboratory.[60] These proteins differ not only in solubility and molecular weight but also in the strength of binding of haem to the apoprotein. The detergents employed were the zwitter-ionic surfactant 1-(*N*-$C_{12}H_{25}$-*NN*-dimethylamino)propane-3-sulphonate (TM 3-12) and the non-ionic surfactants Twin 20 and Triton N-101, belonging to type *A*, which embraces amphiphilic compounds exhibiting lyotropic mesomorphism and forming spherical or ellipsoidal micelles in an aqueous medium.[119] We also used sodium cholate, which belongs to the group of charged detergents of type *B*, differing by the absence of lyotropic mesomorphism and forming aggregates in an aqueous medium containing from 2 to 8 surfactant molecules.[119] The interaction of the haemoprotein enumerated above with detergents of both types is accompanied by a decrease of the intensity of the Soret bands in the absorption spectra. The rate of this process is determined by the nature of the detergents, their concentration, and temperature and is a maximum for the zwitter-ionic surfactant TM 3-12. The rate constants for the transformation of the haemoproteins depend in a complex manner on the detergent concentrations. Their influence on the secondary structure and conformation of proteins, leading to the loss of haem, has been demonstrated by the CD method and the method involving the second derivatives of the absorption spectra of haemoproteins in the presence of detergents. In conformity with the model proposed for the interaction of detergents with haemoproteins, the process takes place at two levels—molecular and supermolecular.[60] At detergent concentrations below the critical concentration for micelle formation, a bimolecular interaction of the surfactants with the proteins is observed; above this concentration, not only surfactant molecules but also their micelles (type *A*) or associated species (type *B*) interact with the proteins. The molecular mechanism of the interaction of surfactants with proteins consist in the fact that the surfactant molecules penetrate via their hydrophobic fragments into

the cavities and "cracks" in the protein molecules, making more hydrophobic the regions adjoining the aromatic aminoacids, which is confirmed by the increase of the amplitude of the second derivatives in the absorption spectra of cytochrome c and P-450 LM-2 following the addition of Twin 20 and TM 3–12.[60] Not only does the hydrophilic–hydrophobic balance change within the protein globule but also the secondary protein structures break down, which is confirmed by CD spectra, reflecting the decrease of the content of the α-helix sections in myoglobin molecules after the addition of TM 3–13.[60] The zwitter-ionic surfactant TM 3–12 interacts most strongly with proteins. The effect of surfactants on haemoprotein leads to conformational changes in the protein, facilitating the loss of haem. The transformation of myoglobin in the presence of detergents of different types takes place with E_{act} = 17–23 kcal mol^{-1}, which can be attributed to the dissociation of the haem iron–histidine nitrogen coordinate bond.[60] Individual detergents and their mixtures increase or decrease the values of ΔH^* for the transformation of myoglobin. A relation (compensation effect) is observed between ΔH^* and ΔS^*, which is described by the equation $\Delta H^* = A + \beta\Delta S^*$, where A = 22 000 cal mol^{-1} and β = 300 K. The coefficient β should usually be close to the average experimental temperature, which has in fact been observed in the present case: $T_{av} \cong \beta = 300$ K.

Inverted surfactant micelles in organic solvents which have solubilised the proteins constitute a special case. Proteins are almost insoluble in pure organic solvents. In order to increase their solubility, certain surfactants are employed. In contrast to aqueous solutions, in the inverted micelles the detergents interact with proteins mainly via polar fragments. A mixed solvation shell consisting of surfactant molecules, the organic solvent, and water is formed around the protein globule. The composition of the solvation shell and the ratio of the components in the micellar solution influence directly the stability and activity of the proteins in the system. The reader can obtain exhaustive information about the functioning and properties of the proteins in these systems from a number of reviews.[83–85,121]

V. TEMPERATURE DEPENDENCE OF THE RATE CONSTANTS FOR THE INACTIVATION OF ENZYMES AND THE PHYSICOCHEMICAL SIGNIFICANCE OF THE THERMODYNAMIC ACTIVATION PARAMETERS FOR THIS PROCESS

The temperature variation of the rate constants for the inactivation of enzymes is described by the Arrhenius equation and is characterised by a single value of E_{act} only if the enzymes have been studied in highly dilute solutions. Only one E_{act} is characteristic of dilute solutions of monomeric lysozyme[47] and subunit lactate and malate dehydrogenases.[58,59] An increase of the protein concentration in solution leads to the appearance of breaks in the Arrhenius plots (Fig.4), since the constants k_{in} depend on the initial protein concentration and other parameters.[47,58,69] In all cases, when the initial protein concentration has been chosen arbitrarily, there are breaks in the Arrhenius plots reflecting changes in the rate constants for the inactivation of enzymes in concentrated solution. For membrane proteins, there are other causes which explain the presence of breaks in the Arrhenius plots: firstly, the conformation and stability of the proteins in the membrane depend on the effect, nature, and physical properties of the surrounding lipids, whose phase transitions induce an abrupt change in E_{act} in the thermal transformation of the protein;[82,90,113,114] secondly, at certain temperatures there is a possibility of "phase" transitions in the protein itself, leading to a sharp change of its conformation[41–44,122] and hence to a change in E_{act} for the thermal degradation process; thirdly, there is a possibility of the existence of different conformers of the same form of the biocatalyst, which are characterised by different E_{act} for the thermal denaturation process. All the possibilities listed above can be realised not only in biomembranes but also in liposomes[123,124] and possibly in inverted surfactant micelles.

Clearly the rate constants for the inactivation of proteins determined in highly dilute aqueous solutions are closest to their true values, because in this case the possibility of the association of the monomeric proteins and of the reassociation of the subunits of oligomeric enzymes is reduced to a minimum. It is therefore useful to consider E_{act} on the activation characteristics ΔH^* and ΔS^* obtained with the aid of the Arrhenius–Eyring equation (1) in the first place for those proteins whose inactivation has been characterised by the rate constants k_0 for their dilute solutions. Under such conditions, we investigated three enzymes–lysozyme,[47] malate dehydrogenase,[59] and lactate dehydrogenase.[58] It follows from the Arrhenius equations (8), (11), and (12) that only in the case of lysozyme [Eqn.(8)] is the pre-exponential factor for the rate constant close to its limiting value for unimolecular reactions.[6,52] For two subunit enzymes, the pre-exponential factor exceeds by many orders of magnitude the typical factors in the equation for unimolecular reaction rate constants (10^{10}–10^{11} s^{-1}) and corresponds to a high activation entropy (Table 1). This is not unexpected, because it has already been known for a long time that the inactivation of proteins is characterised by high values of ΔH^* and ΔS^*.[6–8,10,11,23,125]

In applying the Arrhenius–Eyring equation (1) to the transformations of enzymes and enzymic reactions, one must remember that, as a consequence of the non-elementary nature of these processes, the parameters determined from the temperature variation of k_{in} for enzymes and the rate constants for the enzymic reactions are always real quantities reflecting the entire complexity of the macrostage being characterised—the presence of microstages, which are not outwardly manifested, the influence of the solvent and the environment of the enzyme, etc.[8] (pp.175–185). For this reason, in the analysis of the thermodynamic and kinetic parameters of the inactivation of enzymes, it is essential to resort to additional considerations based on the theory of chemical kinetics and empirical data for simpler chemical and model systems and also to the results of the study of the enzyme inactivation processes by NMR and CD spectroscopic and densitometric methods, sedimentation analysis, etc.

As regards kinetic data, Likhtenshtein proposed three types of methods for their analysis in the case of enzymic processes:[8] (1) quantitative comparison of the energetic and entropic parameters for enzymic and model reactions; (2) theoretical estimation of the parameters for different versions of the mechanism of the reactions investigated; (3) correlation between the energetic and entropic parameters of enzymic processes (compensation effect). Since the present level of the theory does not allow the calculation of the absolute values of the energies and entropies of activation for reactions in a condensed phase, the third type of analysis of enzymic processes is of great interest, particularly when one is dealing with series of reactions, with the specific alteration of one of the parameters keeping the others constant, or with changes in the conditions of a process involving the same enzyme.

The correlation between the energetic and entropic parameters in an enzymic process was first noted by Doherty and Vaslow.[126] On changing the pH, the authors observed parallel compensating changes in the parameters ΔH and ΔS

in the binding of the substrate to α-chymotrypsin. Since then, numerous data concerning the compensating changes in ΔH and ΔS (ΔH^* and ΔS^*) in a wide variety of enzymic processes have been published. These data have been examined in detail and analysed in a series of investigations.[8,11,12,125-130] The survey of the available information about the inactivation of proteins shows that the principal kinetic characteristics of this process are as follows: (1) sharp changes in the rates of inactivation are observed within a fairly narrow temperature range or within narrow ranges of concentrations of hydrogen ions, organic solvents, and other additives; (2) the inactivation is characterised by anomalously large (compared with the parameters of the usual chemical reactions) values of E_{act} and ΔS^*, which reach 180 kcal mol^{-1} and 600 e.u. per mole of the protein in some cases (see Table 1 and also the compilations of data in Refs. 3, 6, 7–11, 22, 23, and 125); (3) the values of E_{act} and ΔS^* are extremely sensitive to changes in the conditions within the medium (pH, ionic strength, nature of the buffer, the presence of stabilising additives, the introduction of proteins into micelles, liposomes, and gels, etc.); (4) there is a linear relation between the experimental values of E_{act} and the activation entropy for different proteins and for the same protein under different conditions, expressed by the equation[131,132] $E_{act} = \alpha + \beta\Delta S^*$.

It follows from the numerous data discussed previously and quoted in this review that, on passing from one protein to another and also on changing the pH and the concentration of ions and denaturing additives, the activation energy and entropy almost always change in parallel (see, for example, Fig. 11). The slope of the linear plots of E_{act} against ΔS^* is known to be the same as the slope of the linear plots of the reversible inactivation parameters ΔH and ΔS.[7-10,22,125] The kinetic and thermodynamic compensation effects are characterised by similar coefficients β and identical scales of the changes in the energetic and entropic parameters.[8,125]

In conformity with existing ideas, the processes involving the inactivation (denaturation) of biopolymers are cooperative conformational transitions. The thermodynamic theory of cooperative systems has been developed in a number of studies in relation to biopolymers.[133-137] Denaturation is sometimes regarded as a "fusion" process, although in the case of proteins one can speak of analogies with fusion processes and "phase" transitions only approximately, since proteins are regarded as "aperiodic" irregular crystals, characterised by differences between the energies of the cooperative linkage and by defects in various parts of large molecules. The cooperative processes in proteins are intermediate between the usual chemical reactions, in which a small number of molecules participate, and large-scale phase transitions with participation of an enormous number of identical species.[8] We shall not consider here the different kinetic models of the conformational transitions in proteins, since they have been analysed in detail in monographs and reviews.[8,10,125,133-137]

According to Privalov,[138] there is at present no doubt about the energetic discreteness of proteins, which reflects their structural discreteness, since proteins are complex systems consisting of more or less independent but fully defined cooperative subsystems. The subdivision of the macromolecule into cooperative subsystems (domains) is a general principle of the structural organisation of protein molecules, which has evolutionary, functional, and physical bases. E_{act} and the activation characteristics ΔH^*, ΔS^*, and ΔG^* for the thermal inactivation (denaturation) of proteins therefore refer to complex cooperative rearrangements of a part of the protein molecule and of its immediate environment. The question arises whether it is possible to refer in some cases the characteristics listed above to a rigorously defined molecular stage in the inactivation of the protein. The set of kinetic,[47] thermodynamic,[41] spectroscopic,[42,45,46] and ^{13}C NMR data[43] obtained for the inactivation of lysozyme permits the claim that this process begins with the ionisation of the Glu-35 residue, which destabilises the adjoining section of lysozymes and causes the breakdown of its α-helix structure in the region of the active centre. Thus, even in this comparatively simple object, which has been characterised in detail, the rate-limiting step in the irreversible inactivation is accompanied by complex conformational changes, which are reflected quantitatively by the values of E_{act}, ΔH^*, and ΔS^*.[47] A second example of a protein for which one can speak quite definitely about the elementary inactivation stage is cytochrome P-450. The loss of catalytic activity by this protein is associated with the increase of the length and rupture of the coordinate bond between the haem iron and the sulphur atom of the cysteine residue.[50,82,88,90] However, a change in the conformation of the apoprotein precedes this step, i.e. in this case the rate-limiting stage in the activation process is associated with marked conformational changes in the biocatalyst, which is reflected by the thermodynamic and activation characteristics of the process.[50,64,65]

The question arises in what cases is it legitimate to calculate the activation characteristics of the thermal inactivation of proteins from the temperature variation of k_{in}, the effective character of which is known beforehand. We believe that this can be usefully done only when one is dealing with a comparison of the behaviour of the same enzyme under the influence of different agents, a comparison of different enzymes acted upon by the same agent under identical conditions, or the modification of the enzyme by various agents under the same conditions. There is no point in discussing the absolute values of E_{act}, ΔS^* and ΔG^* in such cases, since only the character of the variation of these quantities when a specific parameter is varied and all the remaining parameters are kept constant, and not the values themselves, carries information. Thus analysis of the activation characteristics obtained reduces to the discussion of the compensation states between their energetic and entropic components.[7,8,11,125,127-132]

In discussing the enthalpy–entropy compensation in the inactivation (denaturation) of proteins, one should remember that there is a doubt about its existence as an independent physical phenomenon.[9] It has been claimed that, in all enzymic reactions exhibiting the enthalpy–entropy compensation, a key role is played by the solvent (liquid water), which is a constant participant in all physiological processes and constitutes a natural inexhaustible reservoir of chemical free energy in living nature.[125,129] The conformational rearrangement in proteins and the changes in their interactions with water molecules which they entail alter significantly the structure of the entire mass of liquid water. This factor and also the very narrow range of independent parameters (pH, temperature, and the salt composition of the medium), which determine the conditions of the enzymic process, lead to the appearance of trivial linear relations between the enthalpy and entropy changes.[125,129]

Blyumenfel'd[9] quite justifiably assumes that the enthalpy–entropy relations do not reflect any special essential physical features of the reactions and are a simple consequence of the general laws of equilibrium thermodynamics and may not exist at all if the equilibrium thermodynamic process ($\Delta G \cong$ const.) occurs within a very narrow range of variation of an independent variable (temperature, hydrogen ion concentration, etc.). In the study of the reactions of biopolymers, where equilibrium statistical dynamics is applicable only subject to major stipulations, any kind of serious analysis of compensation relations frequently lacks physicochemical significance.

VI. CONCLUSION

It follows from numerous experimental data that the thermal inactivation of enzymes is a complex process consisting of several consecutive-parallel stages and therefore it frequently cannot be described in terms of the first order kinetic law. As a rule, the semilogarithmic forms of kinetic curves have breaks or are altogether non-linear. Even in those cases where a first-order equation describes satisfactorily the kinetic data, the effective inactivation rate constant k_{in} depends on the initial protein concentration.

Two approaches have been proposed to achieve a rapid and correct quantitative characterisation of the process leading to the thermal inactivation of proteins. One of them has been used by Poltorak and co-workers;[24-32] the thermal inactivation scheme is described by equations which have been solved by approximate methods permitting the determination of the rate constants for the first reversible and second irreversible stages and the calculation of the equilibrium constant between the native and reversibly inactivated forms of the proteins. This method permits a quantitative description of the kinetic curves for the thermal inactivation of monomeric and subunit proteins at an arbitrarily selected initial concentration of the latter.

The second method has been used in a series of our own investigations[47,58,59,70] and consists in plotting kinetic inactivation curves for different initial protein concentrations, their linearisation in terms of a first order equation, the determination of the dependence of k_{in} on the protein concentration, and the calculation of the rate constant k_0 at infinite dilution of the protein. We believe that the rate constant k_0 is the most correct quantitative characteristic of the thermal inactivation of proteins. The disadvantages of our method are the approximate character of the equations relating k_{in} to the initial protein concentration and the difficulties in assigning a physicochemical significance to the coefficients α and γ in these equations [see Eqns.(9) and (10)]. An advantage of the method is the fact that we obtain the rate constant for infinitely dilute solutions and hence can apply the equations of classical thermodynamics in the analysis of the temperature variation of these constants. The temperature variation of k_0 is described by the Arrhenius equation, while $k_{in}-1/T$ plots are complicated by breaks and the interpretation of these relations altogether lacks physicochemical significance.

One should recall that the thermodynamic activation parameters (ΔH^*, ΔS^*, and ΔG^*), obtained from the analysis of the temperature variation of k_0, cannot be referred to a single rigorously defined molecular stage in the thermal inactivation of proteins, because they always characterise a complex cooperative process involving conformational transformations in the biopolymer. This is the main cause of the difference between these quantities and the activation parameters for the thermolysis of low-molecular-weight chemical compounds.

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